



# CLINICAL BIOCHEMISTRY



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MELBOURNE UNIVERSITY PRESS

*First edition, 1925*  
*by W. Ramsay (Surgical) Ltd., Melbourne*  
*and republished 1930, 1935, 1938, 1944, 1947, 1950, and 1952*  
*First M.U.P. edition, completely revised, 1956*

*Printed and bound in Australia by*  
*Melbourne University Press, Carlton, N.3, Victoria*

*Registered in Australia for transmission*  
*by post as a book*

*London and New York : Cambridge University Press*

## PREFACE TO THE SEVENTH EDITION

In producing the seventh edition of this book I am greatly indebted to my colleagues in the Departments of Biochemistry and Physiology.

To Professor V. M. Trikojus, in whose Biochemical Department much endocrine research is in progress, I owe my thanks for having rewritten portion of the chapter on endocrine glands and also for his expert advice concerning blood analysis. Professor R. D. Wright has largely rewritten the chapter on water and inorganic electrolytes.

Associate Professor W. A. Rawlinson, Mr J. W. Legge and Mr Ian Parsons have made most valuable contributions to the discussion of haemoglobin and its related pigments and to blood and blood pigments in urine. Dr C. W. Crane, who has made a special study of hepatic disorders, has reconstructed the chapter on liver disease and liver function tests and has added much new material of great interest. Dr J. Bornstein has given his special knowledge in bringing up to date the sections on glycosuria, pancreatic efficiency and acidosis and Dr Vera Krieger of the Royal Women's Hospital has made many suggestions which are incorporated in the discussion of renal efficiency.

I should like to thank Miss M. Bailie and Dr Murray Maxwell for reading the proofs and Miss Bailie also for describing the photoelectric colorimeter and for her work in preparing the index. Others to whom I am indebted for criticism of the text are Miss Beryl Splatt, Dr Alan Mackay and Mr J. Somerset.

Finally, it is a pleasure to express my appreciation of the interest in the book shown by my publishers, the Melbourne University Press.



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# I

## THE INVESTIGATION OF GASTRIC FUNCTION BY TEST MEALS

### INTRODUCTION

Clinical biochemistry has become an essential part of the investigation of many pathological conditions. By its use the diagnosis, prognosis and treatment of disease has been immeasurably improved. The information obtained by clinical biochemistry must be carefully appraised. It is often only one link in a chain of evidence which the clinician assembles and studies before arriving at a diagnosis.

### FUNCTIONS OF THE STOMACH

#### (A) PEPTIC DIGESTION

The glands of the fundus and body of the stomach contain three types of cells:

- (a) parietal or oxyntic cells which are concerned with the formation of hydrochloric acid;
- (b) chief cells which secrete the proteoclastic enzyme pepsin;
- (c) mucous cells found at the neck of the gland tubules and on the surface epithelium of the gastric mucosa and which are engaged in the formation of a mucous secretion.

It is most important to realize that the glands of the pyloric region do not form either hydrochloric acid or pepsin, but secrete an alkaline fluid rich in mucin. In man there is evidence that the body of the stomach and to a less extent the duodenum are the sites of formation of the intrinsic factor of Castle. The glands in the immediate vicinity of the cardiac orifice are of the mucous type and form an alkaline fluid. The secretion of hydrochloric acid and that of pepsin are independent of one another. Hydrochloric acid may be markedly diminished in some circumstances, whilst the pepsin remains normal in amount. Stimulation of the vagus nerves normally causes secretion of a juice rich in pepsin

and strongly acid (0.4 to 0.5 per cent HCl). This is referred to as psychic or appetite juice.

The chemical transmitter of the vagus to the stomach is acetylcholine. The vagi probably control the secretion of mucus by the surface epithelium of the gastric mucosa and the mucous "neck cells". The sympathetic fibres are regarded by most observers as being inhibitory to the peptic and oxyntic cells, but cause secretion of an alkaline mucoïd juice from the pyloric glands. A further factor involved in gastric secretion is a hormone, named gastrin by Edkins which is formed chiefly in the pyloric region and to a less extent in the duodenum and carried in the circulation to the body and fundus of the stomach, stimulating the cells in these areas—particularly the oxyntic cells—to activity (see Fig. 1). Gastrin is a protein of low molecular weight. It is free from histamine and closely resembles secretin in its chemical properties.



FIG. 1. Diagram showing the distribution of the parietal (acid-secreting) cells in the human stomach. In the black area the proportion of parietal cells was maximal and was taken as 100 per cent; in the shaded area on lesser curvature the percentage of parietal cells was 75 per cent, in the dotted area at the fundus 50 per cent, and in the white area 0 to 1 per cent. (After Berger. (1934), *Amer. J. Anat.* 54, 87)

Another factor which influences gastric secretion is the percentage of sugar in the blood. A fall in blood sugar, e.g. following the injection of insulin, stimulates the vagal nucleus and induces

increased gastric secretion of both HCl and pepsin. Gastric secretion is inhibited by a rise in blood sugar.

Fat inhibits gastric secretion, particularly the intestinal phase. It reduces the quantity and acidity of the juice and depresses especially its peptic power. According to Ivy a hormone which has been named enterogastrone is produced in the intestinal mucosa as a result of the action of fat. This hormone when conveyed in the blood to the stomach may cause suppression of gastric secretion lasting from one to four hours and markedly inhibits gastric motility. A hormone which may be named "enterogastrin" is probably produced in the duodenum and plays a prominent part in the stimulation of gastric secretion during the intestinal phase of stomach activity.

Hydrochloric acid acts as an activator of pepsin and the latter converts protein into acid meta-protein, proteose and peptone. If pepsin is rendered inactive owing to the absence of hydrochloric acid, digestion of protein occurs in the small intestine under the influence of trypsin which acts in an alkaline or neutral medium and may complete the hydrolysis to the stage of polypeptides and amino acids.

#### (B) SECRETION OF RENNIN

This is a milk-curdling enzyme and is thought to be formed by the chief cells of the body of the stomach. It converts calcium caseinogenate into calcium caseate, which is insoluble and is responsible for the physical characters of the curd.

#### (C) SECRETION OF GASTRIC LIPASE

This is a weak fat splitting enzyme differing from that secreted by the pancreas in that it acts in an acid medium, the optimum pH being 4 to 5. It ceases to act at pH 2.5. It is probably not of great importance in gastric digestion, but is stated to be of some value in the hydrolysis of highly emulsified fat, such as is found in milk and yolk of egg.

#### (D) SECRETION OF THE "INTRINSIC FACTOR"

According to Castle "pernicious anaemia would not develop if the patient could effect daily the transfer of a millionth of a gram of vitamin B<sub>12</sub>, the distance of a small fraction of a milli-

metre across the intestinal mucosa and into the blood stream". By use of the gastroscope or by gastric biopsy (Wood *et al.*) or at post-mortem examination it is found that the region of the gastric mucosa which is atrophied in pernicious anaemia is that in which the glands normally secrete HCl and pepsin.

Achlorhydria is present in patients suffering from pernicious anaemia and it is common in the relatives of persons suffering from this disease. Complete achylia frequently occurs. It has been shown by Castle *et al.* that 150 ml. to 300 ml. of normal human gastric juice secreted under the stimulus of histamine and then introduced into the stomach of a patient suffering from pernicious anaemia was without effect, as was also 200 grams of beef muscle even after complete digestion with pig pepsin. But when beef muscle and normal gastric juice were given together they produced a marked haemopoietic effect. Apparently there is an interaction between something in normal gastric juice (intrinsic factor) and a constituent (extrinsic factor) in muscle which is responsible for normal haemopoiesis. The extrinsic factor is now known to be B<sub>12</sub> (cyanocobalamin). It is produced by certain bacteria found in the alimentary tract of man and animals and is stored in such foods as meat (muscle), liver and other cellular organs. Gastric juice freed from pepsin and rennin still contained the intrinsic factor. Boiling the gastric juice destroys the intrinsic factor. It is now thought to be a muco-polysaccharide. The intrinsic factor in man is secreted chiefly in the main body of the stomach but perhaps to some extent in the duodenum.

Various theories have been suggested as to the mode of action of the intrinsic factor on the extrinsic factor. The decreased faecal excretion of radio-active cobalt that results from the oral administration of intrinsic factor together with cyanocobalamin (B<sub>12</sub>) suggests that the intrinsic factor facilitates the absorption of the extrinsic factor.

The loss of intrinsic factor in the gastric secretion is a slow and progressive process and the lack of this factor appears to be a quantitative rather than a qualitative effect. The amount of gastric secretion in pernicious anaemia averages about 20 ml. per hour compared with 150 to 160 ml. per hour in a normal person.

Clinical experience in America and Britain indicates that crystalline B<sub>12</sub> given intra-muscularly every three or four weeks

in a dose averaging 1 to 2 $\mu$ g. (1 to 2 microgrammes) daily is adequate therapy. Vitamin B<sub>12</sub> seems preferable to liver extract in pernicious anaemia because it causes less discomfort at the site of injection, does not give rise to untoward reactions and is less expensive.

Hyperchromic macrocytic anaemia may be due to one or more of the following causes :

- (a) Deficiency in the secretion of the "intrinsic" factor. This occurs in pernicious anaemia.
- (b) Deficiency of "extrinsic" factor in the food. This is exhibited in poorly fed people and is common in India. It may be cured by the administration of Vitamin B<sub>12</sub> or by the use of "Marmite" or "Vegemite" which contain the "extrinsic" factor.
- (c) Deficient absorption of B<sub>12</sub> from the alimentary tract manifested in chronic diarrhoea, coeliac disease and sprue.
- (d) Failure of the liver to store B<sub>12</sub> which sometimes occurs in cirrhosis of the liver accompanied by gross hepatic inefficiency.

It is obvious from this summary that hyperchromic anaemia may have a diverse clinical syndrome and the diagnosis can only be accurately determined by careful examination of the blood and of the patient.

#### (E) ANTISEPTIC ACTION

Hydrochloric acid in normal concentration in the stomach destroys many micro-organisms including streptococci which are swallowed with saliva from the mouth and with mucus from the naso-pharynx and tonsils. Diseased tonsils and inflammatory conditions of the maxillary antra and ethmoid sinuses greatly add to the number of bacteria entering the stomach. There is evidence that various infections of the bowels, such as dysentery and typhoid fever, occur more frequently in persons with achlorhydria than in normal individuals. The duodenum is normally comparatively sterile, but in achlorhydria bacillus coli may invade this region from the colon. Chronic diarrhoea is frequently associated with achlorhydria.

## TEST MEALS

The following procedures are in use :

- the one-hour gruel test meal;
- the fractional gruel test meal;
- the alcohol test meal;
- the histamine test;
- the insulin test;
- the combined insulin and histamine test;
- tubeless gastric analysis.

*The One-hour Test Meal*

In this method the patient who has fasted since the previous evening is given at 9 a.m. or some other convenient time a meal consisting of a pint of gruel (for preparation see p. 9). The contents of the stomach are aspirated exactly one hour later and analysed. It should be noted that no aspiration is performed before giving the gruel meal.

The one-hour test meal is open to various objections, the chief of which are:

1. A single analysis at the end of one hour does not give a clear indication of what is happening in the stomach during the preceding 60 minutes, nor does it reveal what may occur during the subsequent period till the natural emptying of the stomach is completed.

2. No information is obtained as to the volume or composition of the fasting gastric contents.

3. Gastric analysis gives figures which are used mainly for comparative purposes, and hence the stomach should be, as far as possible, under identical conditions before the test meal is given. Since the resting gastric contents varies markedly in volume and composition in different individuals, this variable factor should be eliminated by removing the resting juice before the test meal is given, otherwise this residue when mixed with the ingested meal may modify considerably the composition of the material subsequently aspirated.

4. The rate of emptying of the stomach cannot be accurately determined in the one-hour method.

For these reasons most clinicians prefer the fractional method.

*The Fractional Test Meal*

In this method an attempt is made to follow the various phases of gastric digestion. A rubber tube of small bore is passed into the stomach and any residuum present removed. The tube is left in position and the meal swallowed. Every 15 minutes after taking the meal a sample of gastric contents is removed, until the stomach is empty; analyses of the samples are then made.

Ryle has laid emphasis on the following advantages of the fractional method:

1. It enables an accurate study to be made of the fasting gastric contents and allows pathological constituents in this or in subsequent aspirated specimens to be detected.
2. It allows the details of gastric secretion to be followed during the whole stage of digestion, and late rises in acidity and prolonged secretion to be detected.
3. The time at which biliary regurgitation occurs can be noted.
4. The emptying rate of the stomach is determined.

The chief criticisms of the fractional method are:

1. The tube acts as a foreign body in the stomach and may induce abnormal secretion. It has, however, been demonstrated that inert foreign bodies in the stomach do not influence its secretory activity to any marked degree.

2. The tube stimulates excessive salivation, and the saliva, if swallowed, would dilute and partially neutralize the gastric juice. To avoid this, patients are provided with a vessel into which saliva is expectorated.

3. Psychical inhibition of the gastric secretion may occur as a result of anxiety or fear. This does not seem to be a serious criticism, except in a very occasional patient.

4. It is stated that variations occur from day to day in the gastric curve of an individual. This may be so, but the variations are usually of no great magnitude. It has been observed that, in patients on whom the test meal was performed daily for several days, the curve was lower on the first occasion than on subsequent days. If a low curve is obtained in a patient with symptoms suggestive of hyperchlorhydria, it is well to repeat the test next day.

5. It is urged that the gastric contents at any time are not

or coloured yellow or green with bile. Normally a charcoal biscuit given the previous evening should have passed through the pylorus. In pyloric obstruction several hundred ml. may be present containing food residues from previous meals. In gastric ulcer or in gastric carcinoma, blood, either bright red or in the form of coffee grounds, may be present. In carcinoma of the stomach the contents may have a foul odour, and frequently shows achlorhydria, whereas when the obstruction is due to pyloric stenosis there is usually no putrefaction in the food residue as there is normal or hyper acidity which prevents bacterial action. Mucus in excess occurs in chronic gastritis and is recognized by its viscous character. On microscopic examination, the sediment from the fasting juice may show epithelial cells from the pharynx and oesophagus and meat and vegetable fibres occur in cases of pathological retention. Red blood corpuscles, pus cells and various micro-organisms are frequently present in association with gastric ulcer or gastric carcinoma.

(ii) *Samples aspirated during digestion of the meal*

Normally these have the appearance of gruel in suspension and on centrifuging or on sedimentation the supernatant fluid is clear like water. The later specimens are sometimes bile-stained and these samples on standing become green (if not already so) owing to the oxidation of bilirubin to biliverdin. Pathologically, blood, pus, or much mucus may be present.

Filtration through fine filter paper is very slow so that it is better to use either gauze or coarse filter paper for this purpose. In the text is indicated where it is necessary to use filtered or unfiltered specimens for the various tests.

*The Alcohol Test Meal*

In this procedure alcohol is substituted for the gruel used in the method just described. It is widely used in some countries, particularly in Germany, as it is claimed, and justly so, that alcohol is a more powerful stimulus to gastric secretion than is the gruel meal. There is considerable variation in the details of the test, but a frequent procedure is to remove the fasting gastric juice and then to administer 50 ml. of 7 per cent alcohol by the stomach tube. Ten ml. of the stomach contents are then aspirated every 15 minutes until the stomach is empty. The interpre-

tation of the analysis of the aspirated material is similar to that of the gruel meal, but one must remember that the acidity tends to be higher and there are greater variations in the emptying rate of the stomach. The alcohol test meal no doubt illustrates very well the high acidity that tends to develop in many people following the modern custom of taking cocktails before meals, but does not show the modification of acidity that results from the subsequent ingestion of mixed foods (containing protein, carbohydrate and fat) as in an ordinary meal. Whilst it may be maintained that the gruel meal does not stimulate the flow of gastric juice sufficiently, alcohol (by itself) is too strong a stimulus.

### *The Histamine Test*

The resting juice is first aspirated. Histamine is now injected subcutaneously in a dosage of 0.1 mg. per 10 kg. of body weight. The stomach is emptied by aspiration every 10 minutes for one hour or longer if necessary. Each of the specimens is titrated with N/10 NaOH and the free hydrochloric acid and total acidity estimated. These differ by about 10 to 15 units in normal persons. Injection of histamine may cause some throbbing in the head, headache and cutaneous flushing, but these are of temporary duration except in persons with low blood pressure when the dose should be reduced to 0.2 to 0.3 mg. and adrenaline injected if collapse occurs. The volume of gastric juice secreted and the highest free acidity obtained are the most important factors to determine.

The resting juice should be examined as in a gruel meal and the findings have a similar significance as in that test. Usually much higher concentrations of acid are found in the specimens withdrawn in the histamine test than in the fractional test meal. A number of cases which show achlorhydria in response to the ordinary fractional test meal may be found to secrete acid after the injection of histamine. This test is valuable in the diagnosis of pernicious anaemia with or without signs of subacute combined degeneration of the spinal cord. In these conditions achlorhydria is invariable and diminution or absence of pepsin is common.

Histamine injection is frequently used in combination with the fractional test meal. If at the end of an hour no free acid is

found in the fractional meal an injection of histamine is given and this stronger stimulant may cause the secretion of acid. Using histamine as the stimulant, Funder and Weiden have recently correlated test meal findings and the histology of the stomach as shown by gastric biopsy (Fig. 2). They demonstrated that the lesions of superficial gastritis and atrophic gastritis were associated with a depression of hydrochloric acid secretion, more severe with atrophic gastritis than with superficial gastritis. They further showed that all patients with gastric atrophy (those with pernicious anaemia and/or subacute combined degeneration of the spinal cord) had achlorhydria. There was also evidence of profound depression of secretion of pepsin in these patients, though some suffering from pernicious anaemia secreted a little pepsin.

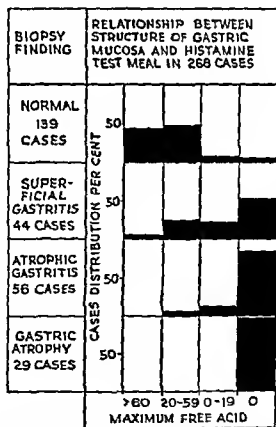


FIG. 2. Showing that there is a close relationship between the histology of fragments of mucosa obtained from the body of the stomach by biopsy and secretion of acid after histamine stimulation. (Funder and Weiden (1952), *Med. J. Aust.*, 1, 600).

### *Augmented Histamine Test*

A. W. Kay has recently introduced an augmented histamine test. In this test he precedes the injection of histamine by an injection of an anti-histamine (anthisan) which antagonizes all histamine effects except that on gastric secretion. In this way a much larger dose of histamine can be used to stimulate the gastric mucosa without causing unpleasant side-effects. He uses continuous aspiration in his test, thus avoiding reduction in total volume aspirated (due to loss through the pylorus) as occurs if the content is removed every 15 minutes by syringe.

### *The Insulin Test*

The subcutaneous injection of insulin (5 to 10 units) causes a marked secretion of gastric juice which is similar to that produced by stimulation of the vagus nerves. Insulin produces this effect by virtue of the hypoglycaemia which it induces. Such hypoglycaemia stimulates the central nervous system and impulses pass via the vagi to the stomach causing a secretion rich in hydrochloric acid and pepsin. If hypoglycaemia is prevented by the administration of glucose then gastric secretion does not occur. This test is of value in determining whether double vagotomy performed for peptic ulcer has been effective. If, after such a surgical procedure, gastric secretion follows the injection of insulin then it is evident that severance of the vagi has not been complete and some fibres are still functioning (Fig. 3).

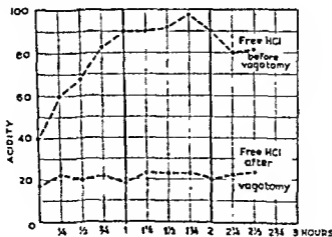


FIG. 3. Insulin Test Meal

*Combined Insulin and Histamine Test*

Ten units of insulin are injected, followed 20 minutes later by an injection of 0.5 mg. of histamine. The response is much greater than that obtained in a fractional test meal. Not only is there vagal stimulation of gastric secretion but there is simultaneously the peripheral chemical action of histamine. The acidity may be as high as 0.1N HCl and even up to 0.15N HCl and the rate of secretion as much as 6 ml. per minute. The maximal response is usually about 60 minutes after injection of the insulin.

*Tubeless Gastric Analysis*

When a cation exchange resin with quinine ions attached is taken by mouth, the quinine ions are liberated in the stomach if free acid be present sufficient to give a pH of 4 or less. The quinine is absorbed and excreted in the urine where it can be estimated quantitatively by its fluorescence in ultra violet light. A urinary excretion of quinine above 30 $\mu$ g. in two hours indicates free HCl while a figure below 20 $\mu$ g. suggests achlorhydria. To stimulate acid production by the stomach an injection of histamine should be given at the time of the administration of the resin. A number of substances which displace quinine from the resin must be avoided for 48 hours prior to the tests. These include calcium, magnesium, aluminium, barium, kaolin and iron, as these ions compete with hydrogen for the resin. Patients with chronic renal disease are not suitable for this test as they may have difficulty in excreting the quinine.

The test is a good indication of the presence or absence of HCl but it does not indicate the exact quantity of free acid present.

**QUALITATIVE ANALYSIS OF GASTRIC CONTENTS**

The qualitative analysis involves tests for:

- free hydrochloric acid;
- lactic acid;
- pepsin;
- blood;
- bile;
- starch;
- mucus.

*Test for Free Hydrochloric Acid*

(Günzberg's test)

*Principle.*—Hydrochloric acid when heated with phloroglucin and vanillin exhibits a red colour, which is not shown by organic acids.

*Reagent.*—Günzberg's reagent (p. 360).

*Procedure.*—Add two drops of the reagent to five drops of unfiltered gastric contents in a porcelain basin and mix. Carefully heat over a low flame or on a water bath till a dry residue remains. In the presence of free hydrochloric acid a red coloration appears, which is most marked round the edges of the residue. This test will detect HCl in as low a concentration as 0.003 per cent. The test need not be performed with all the samples of gastric content, but it is well to test the fasting juice and at least one sample of the aspirated meal series, preferably the one showing the maximum acidity, as determined by quantitative analysis.

*Clinical value of the test.*—Günzberg's test enables one to state quite definitely whether the acidity of the gastric contents is due to mineral acid (HCl) or to organic acids, such as lactic acid.

Free HCl is always absent in pernicious anaemia. Achlorhydria occurs in about 4 per cent of healthy young males and in even a larger percentage of healthy young females. Free hydrochloric acid is absent in a large percentage of cases of gastric carcinoma. In asthma, cirrhosis of the liver, in old people and in debilitating diseases of various kinds, achlorhydria frequently occurs. For further discussion see Achlorhydria (p. 29).

*Tests for Lactic Acid*

## (1) FERRIC CHLORIDE TEST

*Principle.*—Lactic acid reacts with ferric chloride to give a yellow colour. Thiocyanates present in gastric secretion or in swallowed saliva may give a reddish colour with ferric chloride, which obscures the yellow of the lactic acid reaction. Addition of mercuric chloride removes this interfering colour.

*Reagents.*—1. Ferric chloride solution (10 per cent).

2. Mercuric chloride solution (saturated).

*Procedure.*—Introduce into a test tube 5 ml. of filtered gastric

contents and into another control tube an equal quantity of water. To each add two or three drops of 10 per cent solution of ferric chloride. The tube with gastric contents may show a reddish yellow colour, which obscures the true yellow of the lactic acid reaction. To each tube now add about 5 drops of a saturated aqueous solution of mercuric chloride. If lactic acid be present the reddish colour disappears and a yellow colour replaces it, whereas, if no lactic acid be present, the reddish colour disappears without any yellow developing.

A refinement of the test is to use an ethereal extract of the lactic acid, thus:—introduce 5 ml. of strained stomach content into a small graduated separating funnel, add 20 ml. of ether and shake the mixture thoroughly. Now allow the ether to separate and then run out the fluid except the upper 5 ml. of ether. To this ether extract add 20 ml. distilled water and 2 drops of 10 per cent solution of ferric chloride and gently shake. In the presence of 0.05 per cent lactic acid a slight green colour is obtained and 0.1 per cent gives an intense yellow-green.

## (2) UFFELMANN'S TEST

*Principle.*—Ferric carbolate which is violet in colour is converted into ferric lactate (yellow) in the presence of lactic acid.

*Reagents.*—1. Ferric chloride (10 per cent).

2. Carbolic acid (2 per cent)

3. Ether.

*Procedure.*—To about 5 ml. of 2 per cent carbolic acid in a test tube add two or three drops of ferric chloride until a well-marked violet colour is produced. Now add a few drops of filtered gastric juice. A change of colour to canary yellow indicates the presence of lactic acid. A modification of the test is to take a portion of the gastric content, add ether and invert several times, and allow the ether to separate. Lactic acid dissolves in the ether which is decanted into an evaporating dish and placed in a hot water bath (the flame turned out). When the ether has evaporated, dissolve the residue in water and perform the test for lactic acid as described above. It should be observed that lactic acid practically never appears in gastric content if the hydrochloric acid content approaches normal (i.e., above 20 units per cent).

*Clinical value of tests for lactic acid.*—In 1930 Dodds and Robertson demonstrated that the lactic acid found in gastric content was produced by fermentation and was only present when the free hydrochloric acid was very low (below 20 units) or absent. HCl in normal quantity inhibits the action of lactic acid forming organisms. Prior to this work of Dodds and Robertson it was thought by many authorities that the presence of lactic acid was pathognomonic of gastric carcinoma. It is true that lactic acid is found in practically all cases of advanced gastric carcinoma, particularly those which are causing pyloric obstruction, but it is also found in non-malignant conditions where there is pyloric obstruction and retention of gastric content. The presence then of lactic acid is only one link in the chain of evidence indicating a diagnosis of carcinoma of the stomach; the other chemical evidence being the presence of blood with usually a low or absent hydrochloric acid and a foul residual content. The history of the illness and the physical, gastroscopic, and X-ray examinations of the patient are even more important clinically than the examination of the gastric contents.

### *Test for Pepsin*

*Principle.*—Pepsin is a proteoclastic enzyme which will digest fibrin when in a medium of suitable hydrogen ion concentration.

*Reagents.*—1. Fibrin.

2. Hydrochloric acid N/10.

*Procedure.*—Measure 5 ml. of filtered gastric content into a test tube and add to this a small piece of fibrin. The tube is then placed in a water bath at 40° C. for one hour. If pepsin be present and the gastric contents contain free hydrochloric acid, digestion of the fibrin will rapidly take place.

*Clinical value of the test.*—If free hydrochloric acid be diminished or absent, digestion will be extremely slow, or does not occur at all. If now, an equal volume of N/10 hydrochloric acid be added, it will be found that in most cases digestion will readily take place, indicating that pepsin is usually present in cases of hypochlorhydria or achlorhydria, so that it is rarely necessary to prescribe pepsin in these cases. In true achylia gastrica, both pepsin and hydrochloric acid are completely absent, and the fibrin will not be digested on the addition of

hydrochloric acid to the stomach contents unless pepsin be added as well. Achylia gastrica occurs in practically all cases of pernicious anaemia.

### *Test for Blood*

#### BENZIDINE TEST

*Principle.*—(See p. 258).

*Reagents.*—1. Saturated solution of benzidine in glacial acetic acid.

2. Hydrogen peroxide (3 per cent).

*Procedure.*—Some gastric content is filtered through coarse filter paper, a little of the residue on the filter paper is scraped off and a suspension made in about 5 ml. of water. This is boiled to destroy oxidases and then cooled, and to it are added 2 ml. of a freshly prepared saturated solution of benzidine in glacial acetic acid and 0.5 ml. 3 per cent hydrogen peroxide. A blue colour developing within a few moments indicates the presence of haemoglobin. A control test should be made, substituting boiled water for the gastric contents.

*Clinical value of test for blood.*—The benzidine test is thought by some observers to be too sensitive when used in gastric analysis. Minute quantities of blood due to slight trauma caused by the stomach tube are detected, and such a cause for a positive benzidine test must always be considered.

The significance of blood in traces is difficult to assess. It may represent bleeding from an ulcer either innocent or malignant or it may be due to trauma by the gastric tube, oozing from oesophageal veins as in cirrhosis of the liver or from bleeding gums, or it may be due to blood diseases, haemophilia, leukaemia or hypothrombinaemia. Altered blood, "coffee grounds", is usually of more significance than traces of fresh blood, but free HCl in the stomach converts oxy-haemoglobin into "altered" blood (acid haematin) in a few moments. The presence of blood in several of the fractional samples is in favour of the haemorrhage being pathological and not due to trauma of the mucous membrane by the tube.

### *Tests for Bile*

#### (1) FOUCHET'S TEST

Place one drop of gastric content on a white tile and to it add

one drop of Fouchet's reagent. The presence of bilirubin is indicated by oxidation to green or greenish blue, an untreated drop of gastric contents serves as a control.

## (2) VAN DEN BERGH'S TEST

*Reagents.*—1. Alcohol.

2. Van den Bergh's reagent

*Procedure.*—Filter some gastric content through coarse filter paper. By means of a knife scrape a little of the residue from the filter paper and transfer it to a porcelain dish. Extract with 5 ml. of alcohol and filter. To the alcoholic extract add about 1.5 ml. of Van den Bergh's reagent. In the presence of bilirubin a red or reddish-purple colour is obtained. If no free HCl be present in the stomach content then a little must be added before making the alcoholic extract.

*Clinical value of the tests.*—The tests just described are used to detect small quantities of bile. They are unnecessary when bile is present in large quantities since the gastric contents are then yellow or, after standing, green, due to the oxidation of bilirubin to biliverdin. The presence of bile in the stomach in the terminal stages of digestion occurs in about 40 per cent of normal persons and indicates regurgitation from the duodenum. In some patients, bile may be present in almost all samples of the gastric contents aspirated and may be recognized by its yellow or green colour. Such an excess of bile in the stomach indicates either a freely relaxed pyloric sphincter with continuous duodenal reflux or the development early in digestion of a high intra-duodenal tension, so that anti-peristaltic waves force duodenal contents into the stomach. Complete absence of bile at all stages of gastric digestion may indicate pyloric stenosis, or more frequently, a steep descending grade of tension of the alimentary wall, as the food passes from stomach to duodenum, which prevents regurgitation of duodenal contents into the stomach.

### *Test for Starch*

*Principle.*—Iodine reacts with starch to give a blue colour.

*Reagent.*—Iodine solution (2 per cent).

*Procedure.*—One ml. of thoroughly mixed gastric content is removed from each sample and tested individually for the

presence of starch by the addition of a drop of iodine solution. In the presence of starch a blue colour develops. Erythrodextrin gives a red colour.

*Clinical value of the test.*—The iodine starch test is of some value in determining the emptying time of the stomach, which is indicated by the cessation of the blue reaction. The average emptying time for the gruel meal is about two hours. It has this disadvantage that a very small quantity of starch gives a blue colour with iodine and a positive test may persist for a considerable time, though from a practical standpoint the stomach is empty.

A barium meal with X-ray examination gives more accurate information concerning gastric motility.

### *Test for Mucus*

No chemical test is usually used to demonstrate the presence of mucus, but if it is present in excess it will be obvious to the unaided eye. Pouring the stomach contents from one test tube to another will show the presence of "ropy" material, and if there is sufficient mucus it will hang in a festoon from one test tube to another. The mucin secreted by the glands of the stomach and Brunner's glands is not precipitated by acid.

In actual practice an idea of the presence of blood, bile or mucus can be achieved in many cases by inspection and confirmed if necessary by chemical tests.

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## II

# THE INVESTIGATION OF GASTRIC FUNCTION BY TEST MEALS

(Continued)

## QUANTITATIVE ANALYSIS OF GASTRIC CONTENTS

Reference to Fig. 4 will indicate the general form of free hydrochloric acid curve and also the accompanying changes in total acidity, during the course of a normal fractional test meal.

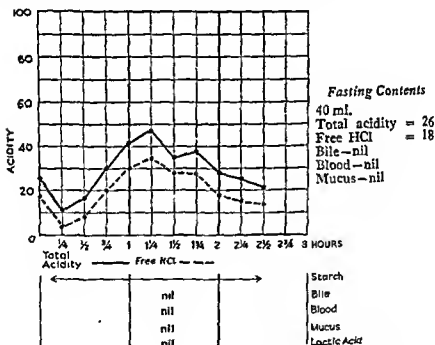


FIG. 4. The range of acidity and the emptying time were within normal limits. No blood, bile or mucus was present. The findings suggest normal function.

The free hydrochloric acid curve shows a preliminary drop due to the dilution of the juice first secreted by the meal introduced into the stomach, but this is usually followed by a steady

rise, till a maximum is reached in about an hour and a quarter, when a gradual fall in acidity occurs till the stomach is empty. The rise in acidity is obviously due to the secretion of hydrochloric acid by the gastric mucous membrane, but the fall in gastric acidity has been ascribed by various authorities to one or more of the following factors:

- (a) Neutralization of the acid by regurgitation of fluid from the duodenum into the stomach.
- (b) Reduction of gastric acidity by combination with swallowed saliva or ingested food and with alkaline mucous secretion of the pyloric region of the stomach.
- (c) Emptying of the stomach.
- (d) Reduction in the *quantity* of hydrochloric acid secreted.
- (e) The secretion of sodium chloride in place of hydrogen chloride (HCl).

The experiments of Boldyreff established the fact of regurgitation into the stomach of pancreatic and intestinal juices and bile. This regurgitation in normal persons, he contended, partly neutralized the acid in the stomach, which he thought was secreted by the gastric mucous membrane in a strength of about 0.4 to 0.5 per cent, so that the hydrochloric acid was finally reduced to about 0.15 per cent or 0.2 per cent. This work of Boldyreff has been supported by various investigators.

Bolton and Goodhart investigated the chloride content of the stomach using the fractional test meal, and demonstrated that as the acid curve rose, the neutral chloride remained relatively low, and after the acid curve had reached its maximum, and begun to fall, the neutral chloride rose, the total chloride curve at this stage remaining approximately constant. They concluded that the fall of the acid curve represented the progress of the neutralization process, which, in its turn probably depended upon relaxation of the pyloric sphincter, permitting duodenal regurgitation. (Part of the neutralization, however, might be due to alkaline mucoid secretion of the pyloric region of the stomach.) The oscillations in the acidity curve which may occur can be explained by intermittent neutralization due to relaxation of the pyloric sphincter and duodenal regurgitation. Again, bile is frequently observed in the stomach contents towards the

end of a fractional test meal in a normal person, and in excessive regurgitation it may be present in all specimens. If there is pyloric obstruction, no regurgitation occurs; the acid curve does not fall, but remains more or less horizontal or climbs owing to absence of neutralization. On the other hand, after gastroenterostomy, achlorhydria may be established by free regurgitation of intestinal contents, although the total chlorides remain the same as before the operation.

Investigations by Bolton and Salmond, by means of X-rays and opaque meals, have shown that in 93 per cent of the cases examined, the duodenum exhibited in varying degrees the movement of anti-peristalsis. This regurgitation seems to be due to an adjustment between intragastric and intraduodenal pressures in which the intraduodenal pressure is temporarily raised above that within the stomach. The tone of the pyloric sphincter and pyloric antrum together control the regurgitation of alkaline material from the duodenum. Although the intragastric pressure during peristalsis may be equal to about 30 ml. of water, or more, the mean intragastric pressure is frequently below 10 ml. of water, whereas the duodenal pressure may rise to 15 or 20 ml., so that regurgitation could occur into the stomach between the gastric peristaltic waves.

As a result of researches by Maclean and his associates, some doubt has arisen as to the importance of duodenal regurgitation in regulating gastric acidity. Maclean constructed an isolated (Pavlov) pouch of the stomach in a dog, with its own nerve and blood supply, but having its contents completely segregated from the main stomach. The secretion in the animal's pouch showed a rise in acid concentration during the digestion of food in the main stomach. This was followed by a fall in acidity, which was accompanied by a rise in neutral chloride, the total chloride remaining approximately constant during the later period of gastric digestion, thus simulating the secretion of the normal human stomach. Since these changes have taken place in a stomach pouch into which no regurgitation of alkaline fluid could have occurred, Maclean contended that the stomach has the power of regulating its own acidity apart from regurgitation.

As a result of these and other experiments, Maclean maintained that the stomach secretes a chloride of about 0.11N

strength. This chloride may be in the form of hydrogen chloride (HCl) or sodium chloride, according to the gastric requirements of the individual. As one rises, the other falls. Towards the close of a meal the hydrochloric acid secretion falls and the sodium chloride rises, the total chloride remaining practically constant. Maclean did not deny that duodenal regurgitation may occur to some extent, but he maintained it plays a very minor part in regulating normal gastric acidity.

Work by MacLagan, and also by Shay, Katz and Schloss, supports the theory of Maclean and it now seems probable that in the normal person duodenal regurgitation as a means of controlling gastric acidity has been overemphasized by the supporters of this view. This does not exclude the possibility that duodenal regurgitation plays some part in regulating gastric acidity in derangement of the stomach and duodenum.

In pathological conditions of the gastro-intestinal tract regurgitation from the duodenum is of frequent occurrence, in some cases occurring throughout the entire test meal.

It is possible that the regurgitated fluid may have a very variable reaction, and in some instances be almost neutral, as the acid from the stomach will have been more or less neutralized by the alkali in the pancreatic secretion and in the bile found in the upper portion of the duodenum. Such regurgitated fluid would have a diluting effect on the gastric content, but practically no neutralizing action.

Oil and fat inhibit the secretion of gastric juice. The less saturated oils are the more efficient, e.g., linseed oil and cod liver oil are more effective than olive oil. Oil also diminishes the motility of the stomach. These inhibitory effects are probably due to the formation in the intestine of a hormone known as enterogastrone, which is conveyed in the circulation to the stomach and depresses its motility and the secretion of gastric juice.

#### (A) ESTIMATION OF FREE HYDROCHLORIC ACID AND TOTAL ACIDITY

*Introduction.*—The components of gastric contents which, together, constitute the total acidity, are:

free hydrochloric acid,  
hydrochloric acid combined with protein,  
acid salts,  
organic acids.

Of these substances the most important is free hydrochloric acid, which ionizes readily and hence has strongly acid properties, whereas the protein-HCl compound, acid salts and organic acids ionize very sparingly, and have weak acidic characters. The amount of free HCl and the total acidity of each sample of gastric content is determined by titrating a measured quantity of the fluid with deci-normal sodium hydroxide, using Töpfer's reagent (dimethylamidoazo benzol) and phenolphthalein respectively, as indicators. Indicators are so-called because they show changes of colour with differing degrees of acidity or alkalinity of the medium in which they are present. Töpfer's reagent is red in the presence of free HCl and becomes salmon and finally yellow when the acid is neutralized (at pH3.9) by sodium hydroxide. The quantity of alkali necessary to cause this change in colour represents the amount of free hydrochloric acid present. To determine the remaining acidity due to the weakly-ionized components of the gastric contents, phenolphthalein is now added, and is colourless in this weakly acid medium. Titration with alkali is continued till all the acid is neutralized (pH8) and the indicator becomes pink. The total quantity of alkali used in the two titrations represents the total acidity of the gastric contents. The term "combined acidity" has been used by clinicians to indicate hydrochloric acid combined with protein, and also in a loose sense to indicate protein-HCl plus acid salts and organic acid. In this book the phrase is not further used, and reference is made only to the determination of free HCl and total acidity.

*Reagents.*—1. Sodium hydroxide (N/10).

2. Töpfer's reagent (p. 361).

3. Phenolphthalein (p. 360).

*Procedure.*—Arrange a series of beakers or evaporating basins on the bench and measure into each 5 ml. of the gastric content, strained through butter muslin, from the specimens aspirated. To each add two drops of Töpfer's reagent. No. 1 is now titrated with N/10 NaOH from a burette till a distinct lemon yellow

appears, which indicates that the free HCl has been neutralized.

Note the number of millilitres of alkali used. Now add three drops of phenolphthalein and continue the titration till a pink colour is permanent, which indicates that the total acid present has been neutralized. Note the total quantity of alkali used. Proceed similarly with the remainder of the series of specimens.

*Calculation.*—Two methods of expressing the acidity are in use:

1. The acid value is represented by the number (referred to as units in clinical work) of millilitres of N/10 alkali, which would be required to neutralize 100 ml. of the gastric contents.

*Free HCl* = No. of ml. N/10 alkali used to produce change in colour of Töpfer's reagent  $\times \frac{100}{5}$ .

*Total acidity* = No. of ml. N/10 alkali used to produce change in colour of phenolphthalein  $\times \frac{100}{5}$ .

2. The acidity may be expressed in percentage of hydrochloric acid.

*Percentage of free HCl* = No. of ml. N/10 alkali used in first titration  $\times 0.00365 \times \frac{100}{5}$ .

*Percentage total acidity* = No. of ml. N/10 alkali used in complete titration  $\times 0.00365 \times \frac{100}{5}$ .

0.365 per cent of acid is the equivalent of 100 in the first method of calculation.

*Clinical value of estimation of acidity of gastric contents.*—Bennett and Ryle investigated the gastric function of 100 healthy students by means of the fractional test meal. (See Fig. 5). They found that the fasting juice varied in quantity from 10 to 150 ml. with an average of 54 ml., and having a free acidity ranging from 0 to 60 units. In 80 of these cases selected to compose the standard for comparison in clinical work, the free HCl in the fasting juice varied from 0 to 22 units. In these standard patients the free HCl rose during gastric digestion to a maximum which varied from 10 to 48 with a total acidity 10 or 15 units higher. If the difference between the two estimations is greater than twenty units it generally indicates that organic

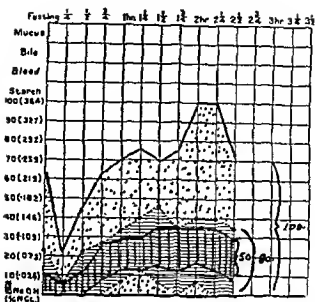


FIG. 5. Showing variations of free acidity in a series of 100 healthy men (Bennett and Ryle)

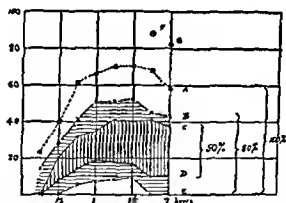


FIG. 6. Showing variations of free acidity in a series of 90 healthy men (Apperly and Semmens)

acids are present which is particularly liable to occur when there is stagnation of gastric contents as in gastric carcinoma or in simple atony. The time at which the maximum acidity developed was one hour and a quarter, and the average emptying time of the stomach was two hours. According to Apperly and Semmens (see Fig. 6) Australian students show slightly higher figures than those found by Bennett and Ryle.

In the investigation of gastric acidity, three types of curve other than the normal isochlorhydria may be found; namely,

those associated with (1) achlorhydria, (2) hypochlorhydria and (3) hyperchlorhydria.

### (1) ACHLORHYDRIA

This term denotes absence of free HCl from the gastric contents, which may be due either to (a) failure of secretion of hydrochloric acid, or (b) its neutralization by combination with alkali or with protein of ingested food or the mucin in gastric secretion. If the former is the correct explanation in any individual case, then the total chloride will be low, while in the second condition it may be normal. The term achylia gastrica should be reserved for the condition in which no true gastric juice is secreted, so that neither HCl nor pepsin are found in the stomach as in most cases of pernicious anaemia. However, the term achylia gastrica is frequently loosely applied to a condition in which no free HCl can be detected, although pepsin is present. This practice, however, should be condemned. As mentioned in the previous chapter, achlorhydria occurs in many pathological states, such as gastric carcinoma (Fig. 7), microcytic anaemia, gastritis (Fig. 8), asthma and in many debilitating

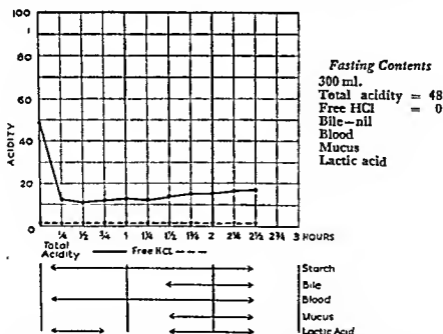


FIG. 7. Achlorhydria. The presence of blood and lactic acid in all specimens and the large volume of fasting contents suggest gastric carcinoma.

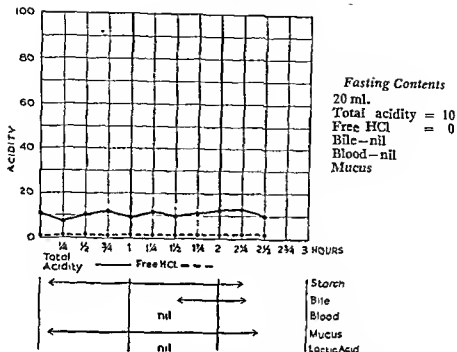


FIG. 8. Achlorhydria. Much mucus was present in all specimens. The findings suggest chronic gastritis.

diseases, and even in 4 per cent of normal young persons (Bennett and Ryle). In such cases pepsin is almost invariably present, and, if the requisite amount of HCl be added to a sample of gastric juice, obtained in the fractional test meal, the pepsin will be found to digest protein quite well. While hydrochloric acid is frequently prescribed for achlorhydria, it is rarely necessary to include pepsin in the prescription.

Apart from the restoration of the gastric mucous membrane by lavage, it has been demonstrated that many cases of achlorhydria were apparent, not real (Fig. 9), since they could be induced to secrete free hydrochloric acid under the influence of a special gastric stimulant such as histamine. (For further details see histamine test meal, p. 11). Using this drug it has been demonstrated that true achylia gastrica is a comparatively rare condition usually associated with pernicious anaemia. Achlorhydria increases with age, as evidenced by a review (Alvarez, Vauzant and others) of 3,381 fractional gastric analyses performed on patients with apparently normal gastrointestinal tracts. The figures show that achlorhydria rose from 4 per cent

at the age of 20 years, to 26 per cent at 60 years of age—the average for the whole series being 12.1 per cent. Females of all ages were more liable to exhibit achlorhydria than males and a strong hereditary or familial tendency to the condition was demonstrated.

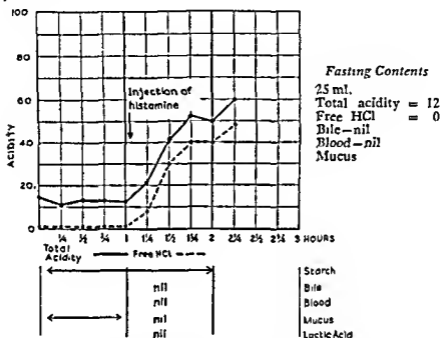


FIG. 9. Achlorhydria for one hour. Secretion of hydrochloric acid followed injection of histamine. The findings indicate false achlorhydria.

## (2) HYPOCHLORHYDRIA

This term denotes diminished quantity of free HCl in the gastric contents, being below 20 units in the above nomenclature. It may be brought about in a variety of ways, and is interpreted somewhat differently by various authors, according to their views of the regulation of gastric acidity. Thus it may be due to:

- Combination of some of the acid with mucus as in chronic gastritis.
- Excessive neutralization of the acid as the result of duodenal regurgitation or by alkaline secretion from the pyloric region.

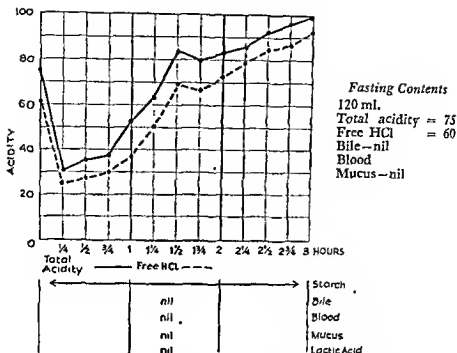


FIG. 11. Hyperchlorhydria. Delayed emptying of stomach. Large quantity of fasting contents. The findings suggest obstructive duodenal ulcer.

which has little stimulating effect on the secretion of acid and the protein of which combines with the free HCl that is secreted; (ii) neutralization of hydrochloric acid by the administration of alkalis; (iii) adsorption of acid by colloidal preparations such as magnesium trisilicate or aluminium hydroxide; (iv) ingestion of olive oil or cream which by stimulating the formation of enterogastrone inhibits the formation of acid; (v) the therapeutic use of atropine or belladonna which lowers acidity by diminishing vagal activity.

(b) *Surgical*. This includes various procedures which aim at: (i) diminishing the secretion of acid by removal of part of the acid secreting area of the stomach, and also the pyloric region which forms the gastric hormone (partial gastrectomy); (ii) facilitating neutralization and emptying of the stomach (gastro-jejunosomy); (iii) removal or exclusion of the ulcer bearing area; (iv) excluding psychic secretion by vagotomy.

As a result of the researches of Bennett and Ryle, Apperley, MacLagan and others on normal persons it may be concluded that neither hyperchlorhydria, hypochlorhydria nor achlor-

hydria can in themselves be regarded as pathological conditions, for the subject of such findings may have no symptoms of gastric disturbance.

### (B) ESTIMATION OF TOTAL CHLORIDES

For details of such estimations larger works may be consulted.

## THE VALUE OF GASTRIC ANALYSIS IN CLINICAL WORK

This has been indicated in the varying sections of this and the previous chapter and now may be briefly summarized as:

1. For detection of cases of achlorhydria.
2. As an aid in the diagnosis of gastric carcinoma and pernicious anaemia.
3. As an aid in the diagnosis of duodenal ulcer.
4. In examining pyloric activity and detecting the presence of mucus or bile in the stomach.
5. To help the surgeon determine whether gastro-jejunostomy should be performed. If the acidity is very high there is considerable risk of post-operative ulcer forming at the stoma.

It must be admitted that the laboratory findings are never pathognomonic of any particular gastric disease, though taken in conjunction with the clinical findings they may be distinctly helpful in determining the diagnosis.

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### III

## LIVER DISEASE AND LIVER FUNCTION TESTS

### INTRODUCTION

As the liver is involved in so many essential metabolic processes of the body, it might be expected that liver disease would alter these activities in such a way as to provide a variety of tests of function. However, in practice, unless widespread necrosis of the parenchymal cells occurs, comparatively few of these well-established processes are sufficiently altered to give much aid in either clinical appraisal or prognosis. This is due to the great functional reserve of the liver. In the dog, up to  $\frac{2}{3}$  of the liver can be resected without death to the animal and with subsequent regeneration of the organ, while in man the occasional successful massive resection of the liver for malignant deposits causes little demonstrable biochemical change. Those tests that are at present widely used in laboratory practice for the investigation of liver disease are mainly empirical in that their mechanisms are not well understood. They are not therefore "function tests" in the same sense that renal function tests investigate specific glomerular and tubular processes within the kidney.

The liver by virtue of its situation in relation to blood supply and drainage may be secondarily involved in pathological changes in the other physiological systems of the body. For example, in consequence of profuse diarrhoea or widespread damage to the mucosa of the gut in such diseases as ulcerative colitis, sprue or regional ileitis, the portal blood fails to obtain from the ingested food the essential nutriments for maintaining normal function, and fatty changes leading to fibrosis can result. Similarly inadequate diets as in alcoholism or chronic starvation produce the same end results, while respiratory, cardiovascular disease or blood diseases may bring about damage to the cells of the parenchyma by resultant anoxia. Obstruction by stones, scar tissue, and malignant invasion of the biliary apparatus, produces back pressure in the liver and so leads to marked pathological

changes. The reticulo-endothelial component of the liver may involve the organ in such widespread conditions as Hodgkin's disease, and blood-borne metastases from malignant deposits elsewhere can lodge in the sinusoids of the lobules and destroy the parenchymal cells by invasion or pressure. Blood-borne parasites, bacteria and viruses (e.g. infectious hepatitis), especially those obtaining entrance to the body via the alimentary tract, can set up acute and sometimes chronic inflammation within the liver and lead to irreversible damage. Certain poisons such as phosphorus, and pharmacological substances such as gold salts and chloroform, may damage the liver preferentially.

The performance of all but the most elementary liver function tests is unwarranted in the majority of straightforward cases of liver and biliary disease seen in general practice, while it is in the case of doubtful etiology or the necessity for added guides to prognosis in the chronic cases that a fuller biochemical examination should be carried out.

Finally, a very careful history together with a full physical examination are pre-requisites when dealing with patients with suspected liver disease, while aids such as cholecystography and liver biopsy often provide key information. Occasionally a laparotomy may be necessary to enable a correct diagnosis to be made.

## THE STRUCTURE AND FUNCTIONS OF THE LIVER

The liver possesses a dual blood supply—arterial from the hepatic artery, supplying about 25 per cent of the blood at a pressure of about 120 m.m. Hg., and venous from the portal vein conveying most of the absorbed products of digestion from the gut and supplying about 75 per cent of the blood at a pressure of about 10 m.m. Hg. The portal vein has an abundant supply of smooth muscle and vasomotor nerves which enable the blood flow to be increased during digestion. Streamlining within the portal vein diverts blood from the stomach, colon and spleen mainly to the left lobe of the liver, while the right lobe receives blood mainly from the small intestine.

The basic unit of the liver is the lobule, 5 to 7 sided with a central vein running through the axis. Classically, the polygonal liver cells are arranged in columns which radiate from the

central vein to the periphery. The columns are separated by the sinusoids, spaces lined with thin sheets of cells down which the portal and arterial blood, mixing at the periphery, passes to the central vein. Fine bile capillaries run between the individual cells. Recent evidence has suggested that the sinusoids are labyrinthine in structure with walls composed of laminae of the liver cells one cell in thickness. Such an arrangement would permit of the maximum surface area for exchange between the cells and the blood. At the periphery of the lobules are the portal triads containing the bile ducts, blood vessels and lymphatics.

When the bile outflow is obstructed either by lesions in the biliary tree or within the liver itself, bile is regurgitated back into the blood stream. It is generally assumed that a rise in pressure within the biliary tree causes the bile capillaries to rupture into the sinusoids. Several workers have failed to demonstrate this in the liver of dogs in which the common duct was ligatured. It may be that the bile reaches the blood merely by diffusion back through the liver cells as the pressure in the biliary tree rises.

Many of the acute hepatic lesions involve the necrosis of some cells in each lobule. Regeneration of these cells restores the normal pattern. Collapse of whole lobules due to fulminating viral hepatitis generally results in their obliteration by fibrosis, while regeneration from areas of surviving cells produces lobules with altered vascular relationships. Fibrosis occurring as a result of this, and active chronic disease processes, tend finally to deprive surviving cells of both nutriment and oxygen, and hepatic failure results. Portal hypertension with the establishment of collateral circulation arises from the disturbances of vascular relationships within the liver.

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*Diseases of the Liver may produce the following:*

(a) Changes in the excretion and metabolism of bile pigments, bile salts, and parenterally administered dyes.

(b) Disordered nitrogen metabolism as shown by decreased serum albumin, prothrombin, and fibrinogen with increased serum globulins; positive empirical turbidity and flocculation tests; abnormal amino acid metabolism; changes in blood ammonia levels.

- (c) Changes in the serum levels of alkaline phosphatase and cholinesterases.
- (d) Alterations in carbohydrate metabolism.
- (e) Alterations in fat and cholesterol metabolism.
- (f) Changes in the electrolyte metabolism and hormone destruction.

★

## (A) BILE PIGMENT METABOLISM

### (1) DISINTEGRATION OF HAEMOGLOBIN

A number of different techniques has established that the life span of the normal human erythrocyte is between 100 and 120 days. In the adult, cells equivalent to about 7 g. of haemoglobin are destroyed daily. Although a vast amount of work has been carried out, the manner in which the first steps in the breakdown are achieved is still largely conjecture.

Earlier theories suggested that haemoglobin from red cells, phagocytosed at the end of their life span by the cells of the reticulo-endothelial system, was degraded by splitting off the globin, removing the iron from the resultant haematin, and opening the remaining porphyrin ring by oxidation to form biliverdin. The iron was stored as a hydrated oxide for re-use in haemoglobin synthesis while the biliverdin was reduced to hilirubin and excreted into and transported by the blood stream to the liver.

The intracellular breakdown was supported by the failure to demonstrate circulating haematin in the blood of normal persons, while the detection of biliverdin in macrophages surrounding areas of bruising and in extracts of tissue cultures following red cell phagocytosis lent support to the view that biliverdin formation probably preceded bilirubin.

Lemberg and his associates, unable to produce evidence that porphyrins were intermediates in this chain of events, proposed an alternative mechanism. They found that haemoglobin in the presence of ascorbic acid could be oxidized by oxygen to give a green pigment with characteristic physical properties under conditions sufficiently mild that the reaction could theoretically take place within the red cell itself. This pigment, which they termed choleglobin, was shown to have retained its native globin

and iron, but the porphyrin ring had now been opened by the loss of a  $=CH-$  group bridging two adjacent pyrrole rings.

Reactions characteristic of compounds possessing porphyrin structure could not be obtained with choleglobin. However, these could be elicited when the porphyrin structure was reconstituted by closure of the ring by treatment with ammonia. Choleglobin was found to part with its iron under mild conditions and form biliverdin readily. Although compounds resembling choleglobin have been shown to be present in the red cells of rabbits, there has yet been no satisfactory demonstration of such compounds in human red cells under normal conditions.

It is probable that there is more than one route of breakdown. Choleglobin might easily be formed in the reticulo-endothelial system and its subsequent degradation with loss of globin and iron, and the conversion of biliverdin to bilirubin, are most likely to take place there.

Studies with haematin into which isotopically labelled nitrogen ( $N^{15}$ ) has been incorporated have shown that this compound might still be an intermediate since it is rapidly metabolized to bile pigment.

## (2) THE PLASMA BILE PIGMENTS

Van den Bergh in 1913 demonstrated qualitatively that bilirubin in normal serum reacted promptly with Ehrlich's reagent (diazotised sulphanilic acid) in the presence of alcohol to give a red condensation product. A similar prompt reaction, in spite of the inadvertent omission of alcohol while applying this test to a specimen of jaundiced serum from a patient with mechanical biliary obstruction, led him to the significant discovery that cases of jaundice could be divided into two groups on the basis of the reaction of sera with Ehrlich's reagent:

(1) the haemolytic type of jaundice requiring alcohol for immediate colour production, the "indirect reaction", and

(2) the purely obstructive type of lesion not requiring alcohol for immediate colour production called the "direct reaction".

Van den Bergh suggested that there were two different pigments responsible for these reactions.

Apart from the reticulo-endothelial component, the main role

of the liver in bile pigment metabolism was deemed to be the removal from the blood and excretion into the bile of bilirubin. The possibility of mechanical rupture of the minute bile canaliculi by back pressure when the biliary tree was obstructed, offered an explanation for the appearance in the blood stream of bilirubin that had in some way been altered by the passage through the parenchymal cells. On the other hand, with an unobstructed biliary tree, bilirubin from haemolytic or normal sera would give the indirect reaction since alteration by the liver cells has not yet occurred. These two pigments became labelled "cholebilirubin" and "haemobilirubin".

Firm attachment in some way to the plasma proteins would explain elevated serum levels in haemolytic jaundice in the absence of urinary bilirubin with altered affinity on passing through the liver. Human bilirubin has been found to migrate with the albumin and  $\gamma$ globulin fractions of the serum proteins on electrophoresis in an identical manner irrespective of the type of jaundice or the type of Van den Bergh response. Other workers postulated inhibiting or catalysing substances to explain Van den Bergh's findings.

The position became further complicated on finding that sera from cases of viral hepatitis could exhibit both types of Van den Bergh reaction.

The observation that bilirubin obtained from normal serum and from patients with haemolytic anaemia was more soluble in chloroform than that from cases of obstructive jaundice led Cole and Lathe (1953) to separate, by chromatography of sera from jaundiced patients, two pigments giving the direct and indirect reactions. The indirect reacting pigment, soluble in chloroform and identical in its behaviour in all respects with pure bilirubin, was isolated from a number of normal and haemolytic sera. The direct reacting pigment, soluble in polar solvents, is thought to be a mixture of two closely similar substances formed from bilirubin in the liver. It was isolated in quantity from the sera of patients with obstructive jaundice. In comparison with pure bilirubin, the solubility in water of the direct acting pigments is striking. This accounts for the appearance of bilirubin in the urine in cases of obstructive jaundice when the serum levels are only slightly raised.

### *Quantitative Van den Bergh Reaction*

The quantitative Van den Bergh test is of undoubted value and is performed in the following manner.

*Principle.*—The reddish violet alcoholic solution of azobilirubin is compared with a standard colour solution, either of ferric thiocyanate or cobalt sulphate in a colorimeter.

*Reagents.*—1. Diazo reagent (p. 357).

2. Alcohol (95 per cent).

3. Saturated ammonium sulphate.

4. Standard cobalt sulphate solution (p. 357).

*Procedure.*—To 1 ml. of plasma add 0.5 ml. of diazo reagent. After two minutes, add 2.5 ml. of 95 per cent alcohol and 1 ml. of a saturated solution of ammonium sulphate. Mix and centrifuge. A layer of clear ammonium sulphate solution is left at the bottom of the centrifuge tube; above this is a white layer of precipitated protein, and super-imposed on this is the clear reddish violet alcoholic solution of azobilirubin, the dilution of which may be taken as one in four. The precipitate of protein does not carry down the pigment if the diazo reagent be first mixed with plasma to allow "coupling" to take place, and then alcohol and saturated ammonium sulphate be added. The intensity of colour of this azobilirubin solution (allowing for its dilution of one in four) is determined quantitatively by comparing it in a suitable colorimeter with a standard, either of ferric thiocyanate (p. 359) or of cobalt sulphate (p. 357). The dilution of the serum in the preparation of the supernatant fluid is 1 in 4, and not 1 in 5 because the saturated ammonium sulphate remains as a separate layer (1 ml.) and does not contain azobilirubin. Thus  $4S/U =$  units of bilirubin where S and U represent colorimetric readings of standard and unknown solutions. The standard solutions have a colour equivalent to one unit of bilirubin (a solution of bilirubin of 1 in 200,000). If the colour of the supernatant alcoholic solution is too intense for immediate quantitative estimation, it is further diluted with alcohol (two parts alcohol and one part water), the dilution being allowed for in the subsequent calculation. One Van den Bergh unit corresponds to 0.5 mg. bilirubin/100 ml.

This procedure measures total bilirubin. Normal total serum

bilirubin values are to 1 mg. per cent while latent jaundice occurs between 1 and 2 mg. per cent. Values over 3 mg. per cent are usually clinically recognizable. The detection of latent jaundice is useful in diagnosis of infectious hepatitis, cirrhosis and haemolytic jaundice. If in jaundiced patients more than half the total bilirubin values are made up of the indirect form then haemolytic conditions are most likely, while if more than half the value is due to the direct form, obstructive lesions or hepatitis are most likely.

### (3) DEGRADATION OF BILE PIGMENTS IN THE ALIMENTARY CANAL

For many years, it was thought that compounds such as urobilin and urobilinogen were formed exclusively as a result of reduction of bilirubin by bacteria in the alimentary tract and that this bilirubin came from one source only, haemoglobin.

Later it was discovered that patients with obstruction of the biliary tree complicated by infection with *B. coli* could produce a urobilin under conditions precluding alteration of bilirubin by passage into the gut, but that this urobilin differed from that found in other pathological conditions. Very recently, normal patients fed with N<sup>15</sup> labelled glycine (which becomes rapidly incorporated into the porphyrin ring of haemoglobin) showed a disproportionately large output of labelled faecal bile pigment early in the experiment conflicting with the widely-held views of a 100-120 day span of the normal red cell. This finding has been confirmed in a number of laboratories and has been shown to occur to an even greater extent in some haemolytic diseases and porphyrias. The most likely explanation is that not all the porphyrin so formed is required for haemoglobin synthesis and that a proportion is degraded straight to bilirubin.

Much confusion has existed in the past on the mechanisms of transformation of bilirubin under normal and pathological conditions, firstly because of the lack of agreement on nomenclature and secondly by the instability of the intermediate compounds. Early workers obtained from pathological urines a strongly laevorotatory red pigment giving a brilliant green fluorescence with zinc salts which they called "urobilin". Its precursor, giving a magenta colour with Ehrlich's aldehyde reagent, and from which it could be obtained by mild oxidation or standing

in aqueous solution, was isolated and called "urobilinogen". Meanwhile a red pigment called "stercobilin", identical in all respects with "urobilin", was obtained in pure form from normal faeces although its precursor, labelled "stercobilinogen" and which was also known to give a reaction with Ehrlich's aldehyde reagent, could not be isolated. The reduction in vitro of pure bilirubin with sodium amalgam yielded a colourless compound called "mesobilirubinogen" which was identical in every way with the "urobilinogen" from pathological urines. On mild oxidation of this compound a rather unstable optically inactive compound called "urobilin IX $\alpha$ " was obtained and not the expected laevorotatory urobilin.

The discovery in bile, from patients suffering with infection of the biliary tree, of a urobilin that was strongly dextrorotatory although possessing all the other characteristics of stercobilin and urobilin IX $\alpha$  added a further complication. Recently this pigment has been isolated from the faeces of patients undergoing aureomycin or terramycin therapy.

Present information supports the scheme below (Fig. 12) that the precursors of all the urobilins, i.e. stercobilinogen, mesobilirubinogen and d-urobilinogen, are formed in the gut under normal conditions in varying quantities and that when pathological urines give reactions for "urobilinogen" they do in fact contain stercobilinogen and mesobilirubinogen. Similarly both stercobilin and urobilin IX $\alpha$  make up the urinary "urobilin". The failure of the early workers to isolate and identify stercobilinogen and urobilin IX $\alpha$  was due to the inherent instability of these compounds.

The formation of d-urobilinogen and its conversion into mesobilirubinogen has been proved. The means whereby the

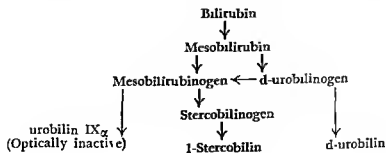


FIG. 12

antibiotics or bacteria are able to form d-urobilin from this compound instead of the normal urobilins is at present unknown.

It is certain that stercobilinogen and mesobilirubinogen are normally absorbed from the gut and pass into the portal vein into the liver. Opinions differ as to whether they are re-excreted into the bile as bilirubin or are completely broken down. Chemical considerations are against oxidation back to bilirubin.

Under normal conditions about 250-300 mg. of bilirubin are produced daily by an adult from haemoglobin breakdown. About 240 mg. of "stercobilinogen" is considered a normal daily output.

The modern work on bile pigment metabolism makes for a more comprehensive understanding of the various types of jaundice.

#### (4) JAUNDICE

This may be classified as follows :

(a) retention or haemolytic jaundice, and

(b) regurgitation jaundice;

(i) mechanical, due to extrahepatic obstruction;

(ii) parenchymatous, due to intrahepatic obstruction arising from pathological changes within the liver.

(a) *Retention Jaundice* is due to the overproduction of bilirubin from excess breakdown of erythrocytes such that the liver is unable to maintain normal blood levels. This bilirubin is composed of the indirect form only. Poor water solubility of this compound may account for the absence of bilirubin in the urine. The faeces contain amounts of stercobilinogen and mesobilirubinogen in excess of 250 mg. daily and are highly pigmented. Reabsorption of increased amounts of these pigments into the portal vein exceeds the capacity of the liver to deal with them and they therefore spill over into the urine, giving positive tests for both "urobilinogens" (stercobilinogen and mesobilirubinogen) and their oxidation products "urobilins" (stercobilin and urobilin  $1x2$ ), but no bilirubin.

(b) *Regurgitation Jaundice.*

(i) *Mechanical Obstruction.* Bilirubin altered by passage through the liver cells to the direct acting form is thought to be

regurgitated back into the blood stream by way of the sinusoids, either on rupture of the bile canaliculi due to increase of pressure within the biliary tree, or by simple diffusion out of the cells. The water-soluble direct type permits of excretion of bilirubin into the urine. Since a diminishing amount of bilirubin reaches the gut, less stercobilinogen and mesobilirubinogen are formed and the stools become pale. Less pigment is reabsorbed into the portal vein, while the liver cells are able to clear this diminished amount. Therefore the urine contains neither "urobilins" nor "urobilinogens".

If infection of the biliary tree occurs, however, the urine will contain, besides bilirubin, the dextrorotatory form of urobilin.

(ii) *Parenchymatous*. There are two mechanisms at work here:

(1) The obstructive element due to intrahepatic obstruction of the biliary tree; and

(2) the failure of the liver to convert the indirect to the direct form of bilirubin and to deal with the diminished amount of reabsorbed "urobilinogen", owing to damage of the parenchymal cells.

In this situation the Van den Bergh test gives both direct and indirect values, the stools are pale, but the urine is found to contain bilirubin, "urobilin" and "urobilinogen".

It is obvious that if the obstructive element predominates then the Van den Bergh estimation will show mainly the direct form, while the stools will be pale and large amounts of bilirubin will be found in the urine.

If, on the other hand, parenchymal damage is uppermost this will be reflected by the increase in urinary "urobilinogen" and "urobilin". The Van den Bergh test will show an increased indirect value and the stools will be pigmented.

Chemotherapeutical substances capable of suppressing the growth of bacterial flora in the gut also depress the formation of stercobilinogen and mesobilirubinogen. Therapy should be checked when daily estimations are being made. Constipation results in the increased absorption of faecal bile pigments, often in excess of the amount the normal liver can handle. Small amounts of "urobilinogen" appear in the urine. This should be borne in mind when tests for suspected liver disease reveal this

as the only abnormality. Normally less than 4 mg. of "urobilinogen" are excreted in the urine daily.

### *Tests for Bile Pigments in Urine*

#### (I) BILIRUBIN

##### (i) *Fouchet's test*

*Principle.*—Bile pigment is adsorbed on to a precipitate of barium sulphate. Ferric chloride oxidises the bilirubin to biliverdin (green) and cholecyanin (blue).

*Reagents.*—1. Barium chloride solution (10 per cent).

2. Fouchet's reagent (p. 359).

3. Magnesium sulphate (sat.).

*Procedure.*—To 10 ml. of urine add 2 drops of saturated solution of  $MgSO_4$  and 5 ml. of 10 per cent  $BaCl_2$  solution. Filter. Transfer and spread some precipitate on a second dry filter paper. Add 2 drops Fouchet's reagent. A green or greenish-blue colour indicates the presence of bile in the urine. This is a very useful test.

##### (ii) *Cole's test*

*Principle.*—The bile pigment in the urine is carried down by adsorption on a precipitate of barium sulphate formed by the addition of magnesium sulphate and barium chloride to the urine. The adsorbed pigment is oxidised by means of sulphuric acid and potassium chlorate with the formation of a greenish-blue colour.

*Reagents.*—1. Magnesium sulphate (saturated).

2. Barium chloride solution (10 per cent).

3. Sulphuric acid (conc.).

4. Potassium chlorate solution (15 per cent).

5. Alcohol (97 per cent).

*Procedure.*—Boil about 15 ml. of urine in a test tube. Add 2 drops of a saturated solution of magnesium sulphate. Then add a 10 per cent solution of barium chloride drop by drop, boiling between each addition. Continue to add the barium chloride until no further precipitate is obtained. Allow the tube to stand for a few minutes. Pour off the supernatant fluid as cleanly as possible. To the precipitate add 3 ml. of 97 per cent alcohol, 2 drops of strong sulphuric acid and 2 drops of a 15 per cent

aqueous solution of potassium chlorate. Boil for half a minute and allow the barium sulphate to settle. The presence of bile pigments is indicated by the alcoholic solution being coloured greenish-blue.

## (2) UROBILIN

### (i) *Bogomolov's test*

To 10 ml. urine add 10 drops of 20 per cent  $\text{CuSO}_4$ . Now add about 4 ml. chloroform; place the thumb over test tube and invert several times without shaking. The chloroform will be coloured yellow if urobilin or urobilinogen be present in excessive amount. Remove by pipette the chloroform and put into a dry porcelain basin—the chloroform clarifies as a pink fluid.

### (ii) *Schlesinger's test*

*Principle.*—Solutions of urobilin appear yellow by transmitted light but green by reflected light.

*Reagents.*—1. Alcoholic solution of iodine (0.5 per cent).

2. Acetic acid (20 per cent).

3. Saturated alcoholic solution of zinc acetate.

*Procedure.*—Acidify about 10 ml. of urine with 4 or 5 drops of 20 per cent acetic acid. Then add 3 or 4 drops of alcoholic iodine (0.5 per cent) and about 5 ml. amyl alcohol. With the thumb placed over the end of the test tube invert it several times. Allow to stand for a few minutes and decant the upper layer. To the decanted fluid add an equal volume of 10 per cent alcoholic zinc acetate. A green fluorescence indicates the presence of urobilin, and this solution will show a spectroscopic band at F.

## (3) UROBILINOGEN

### (i) *Ehrlich's aldehyde test*

*Principle.*—Urobilinogen forms with paradimethylaminobenzaldehyde in acid solution, a red condensation product.

*Reagent.*—Ehrlich's reagent (3 per cent solution of paradimethylaminobenzaldehyde in 20 per cent HCl).

*Procedure.*—To 5 ml. of urine add 2 drops of the Ehrlich's reagent. A deep red colour indicates pathological amounts of urobilinogen. It may be necessary to warm the tube and allow it to stand a few minutes before the colour develops if the amount of urobilinogen is small. Wallace and Diamond have

applied Ehrlich's test to the quantitative estimation of urobilinogen in urine.

### *The Bromsulphthalein Test*

Bromsulphthalein, a dye, is excreted by the liver cells into the bile in a manner analogous to bilirubin. The dye can be readily estimated in the serum owing to its intense colouration with alkalis. If a standard amount of dye is injected into the blood stream, measurement of its rate of disappearance gives some indication of the functional capacity of the liver cells.

A number of control estimations show that at a dosage of 5 mg./kg. body weight, less than 5 per cent retention of dye occurs after 45 minutes. The test must be carried out on fasting subjects. 1/v injection of the dye on the basis of 5 mg./kg. body weight is made into one arm, while blood is withdrawn after 45 minutes from the other arm and allowed to clot. Photocolorimetric estimation against standards is made with the serum treated with N/10 NaOH and the results expressed as a percentage of retained dye. There is a negligible loss of dye in tissue spaces and in the reticulo-endothelial system. The test is little used when clinically recognizable jaundice is present. When liver disease is suspected in the absence of jaundice and the rest of the liver tests fail to reveal much abnormality, the bromsulphthalein retention gives a good indication of the excretory capacity of the liver cells. The test is especially useful in helping to determine whether a patient with severe viral hepatitis should commence convalescence, and in the follow-up of such cases. It is also of great help in the diagnosis of primary liver disease in patients presenting with hepatosplenomegaly. The test does not distinguish between extra and intrahepatic obstruction.

### (B) BILE SALT METABOLISM

Bile salts are synthesized in the liver and excreted into the alimentary canal in the bile where they assist in fat absorption. They are absorbed and re-excreted by the liver and constitute an enterohepatic cycle. When biliary obstruction prevents the excretion of bile salts into the alimentary canal, steatorrhoea results. Vitamin K, a fat-soluble compound necessary for the formation of prothrombin, is not absorbed. Haemostasis is there-

fore impaired. Bile salts appear in the blood and urine in biliary obstruction, but levels tend to fall when parenchymal damage results in impaired synthesis.

### *Tests for Bile Salts in Urine*

#### *(i) Hay's test*

*Principle.*—Bile salts have the power of lowering the surface tension of the fluid in which they may be dissolved.

*Reagent.*—Sulphur in the form of dry fine powder.

*Procedure.*—Take two test tubes (a) and (b). The tube (a) is half filled with water and (b) with the urine under observation. Sulphur in the form of a fine powder is sprinkled on the surface of the fluid in each test tube. The tubes are gently agitated and if bile salts are present a shower of fine particles of sulphur is observed to fall to the bottom of the tube (b). In large doses, sandalwood oil, cubebs, turpentine, copaiba and hexyl resorcinol lower surface tension of urine and enquiry should be made as to their use by the patient. Albumin also lowers the surface tension of urine.

It is preferable to have the sulphur in a container with a perforated outlet at a standard height (1 inch) above the surface of the urine. With a concentration of 1 in 10,000 of bile salts the sulphur will sink instantly, but there is a slight delay with the concentration of 1 in 40,000.

### (C) NITROGEN METABOLISM

It is of interest that one of the main functions of the liver, to form urea from amino nitrogen, shows little impairment except in cases of widespread necrosis of the liver cells as seen in severe infectious hepatitis and chloroform or phosphorus poisoning. Urea output may fall but the blood urea falling initially may rise again owing to renal involvement in these conditions. The effect on protein metabolism on the other hand is often marked.

#### (1) ALBUMIN AND GLOBULINS

Albumin is synthesized only by the liver cells, so that a decrease in the mass of the liver or impairment of synthesis due to disease results in low serum values. Globulins on the other hand, apart from prothrombin and fibrinogen, are related to the mesenchymal elements of the liver. Acute and chronic inflam-

matory processes irritate the stroma of the liver causing formation of fibrous tissue with the rise of globulins. General systemic diseases involving the reticulo-endothelial system such as Boeck's sarcoidosis also cause alterations of the stromal elements with increase of globulins.

A fall in albumin with an accompanying rise in the globulin fractions, particularly the  $\gamma$  globulin, is a well recognized feature of sub-acute and chronic liver disease. When low albumin results from a decrease of cell mass only, such as replacement by neoplasms or in chronic congestion, and inflammatory elements are absent, then these low values are seldom accompanied by rises in the globulin fractions.

Low albumin values are prognostic. Values less than 2 g. per cent indicate severe liver disease with a poor prognosis and are generally accompanied by obstinate peripheral oedema and ascites. Transfusion of albumin in these cases produces a temporary rise only with a fall to the initial level after a few weeks. It seems that in chronic liver disease there may be alteration in the balance between the anabolic and catabolic activities. On the other hand high globulin values with relatively normal albumin levels cause little disturbance, and patients may be in good health. Infectious hepatitis nearly always results in a slight rise of  $\gamma$  globulin which persists for a very long time after clinical recovery.

The changes in the serum proteins were first extensively studied by the moving boundary electrophoresis technique. Paper electrophoresis provides a simple qualitative picture of these changes, and readily shows marked falls of albumin and rises of  $\gamma$  globulin. The protein fractions may be estimated by the Biuret or the Kjeldahl methods using a 23 per cent solution of sodium sulphate as the globulin precipitate (see Plate I).

### *Flocculation Tests*

A number of flocculation and turbidity tests has been devised for the detection of active liver disease. Three of them have been proved to be of great use; the cephalin cholesterol test, the thymol turbidity test, and the zinc sulphate test. The mechanism of flocculation or turbidity differs slightly from test to test and each therefore measures changes in a different component of

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the serum. The routine employment of three tests ensures that these changes reflecting liver damage are not overlooked.

### (1) THE CEPHALIN CHOLESTEROL TEST

This test depends upon the attachment of  $\gamma$  globulin of the serum to particles of a cephalin cholesterol emulsion to produce flocculation. The extent to which the serum is diluted (1:26) for the test, allows of sufficient  $\gamma$  globulin in practically all sera to produce flocculation, but in normals this is prevented by the stabilizing effect of other components, albumin+the lipid rich  $\alpha^1$  globulin being mainly responsible. Old sera in which the albumin- $\alpha^1$  component has deteriorated, or greater dilutions (1:80) of fresh normal sera, will also give a positive result. These stabilizing components disappear from the serum within about 48 hours of acute hepatic injury and remain absent until healing is well established. Flocculation can occur in non-hepatic diseases such as lupus erythematosus, sub-acute bacterial endocarditis, and diffuse diseases of the reticulo-endothelial system where abnormal  $\gamma$  globulin as well as alterations in the  $\alpha^1$ -albumin components are found. The test is therefore not specific for liver disease.

The test is very useful in determining hepatic injury in the absence of jaundice, e.g. in the pre-icteric stage of infectious hepatitis. It indicates acute necrosis of the liver cells and is therefore generally positive in chronic as well as acute hepatic disease. The healing with fibrosis of chronic lesions is usually accompanied by a change from a positive to a negative reaction.

The test is negative in obstructive lesions of the biliary tree, and in space-occupying lesions such as secondary carcinoma unless parenchymal damage has supervened.

*Reagents.*—1. Cephalin-Cholesterol. Stock solution in ether (p. 365).

2. Cephalin-Cholesterol emulsion (p. 365).

3. 0.85 per cent saline (prepared on the day of the test).

*Procedure.*—The test is performed in the standard type of 15 ml. conical centrifuge tube. The tubes must be clean and free from traces of strong acids and alkalis. Four ml. of 0.85 per cent saline and 0.2 ml. of the patient's serum are placed in the tube,

followed by 1 ml. of cephalin-cholesterol emulsion. After mixing thoroughly, the tubes are protected from dust and allowed to stand for 48 hours in a cupboard away from strong light. In reading the test the intensity of reaction is judged by the degree of flocculation. The strongest reaction, designated four-plus, shows complete flocculation, the supernatant fluid over the deposit being completely clear. A negative reaction shows no flocculation, the fluid appearing perfectly homogeneous. Reactions of less intensity than four-plus are judged on the basis of the quantity of deposit and the degree of opacity of the supernatant fluid.

It is essential that the cephalin used be "ripened" by standing exposed to the light and air for six weeks. Stock solutions made from unripened cephalin will result in emulsions which are precipitated by the sera of normal individuals, hence negative control tests are desirable on each set of tests.

Sera must be tested on the day of collection. Keeping even in a refrigerator enhances the flocculating power. Haemolysis must be avoided.

In infectious hepatitis a 1+ to 2+ reaction may occur in the early, rising to four-plus in the acute, stages. In chronic diseases such as cirrhosis, the extent of the reaction parallels roughly the activity of the disease process. In healed lesions it may be negative or weakly positive.

## (2) THE THYMOL TURBIDITY AND FLOCCULATION TEST (MACLAGAN)

This test involves the addition of serum to a buffered saturated solution of thymol. The mechanism of flocculation with abnormal sera differs from that of the cephalin cholesterol in that the phospholipid component of the  $\beta$ -globulin fraction is necessary for the reaction. Although necessary also,  $\gamma$  globulin plays a less defined role. Positive reactions with abnormal sera can be inhibited with normal human albumin. Albumin from patients with viral hepatitis is much less effective. The test is positive in active liver disease whether acute or chronic but becomes so at a later stage than the cephalin cholesterol test. It often remains positive after an attack of viral hepatitis when the cephalin test and the clinical picture have returned to normal, while it may be strongly positive in cirrhosis when the cephalin is but faintly so.

NORMAL

Urine.

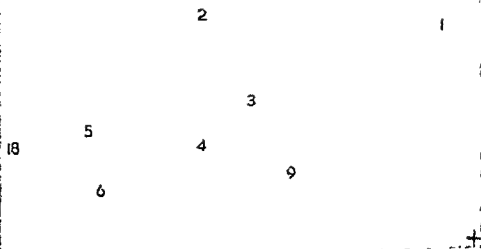
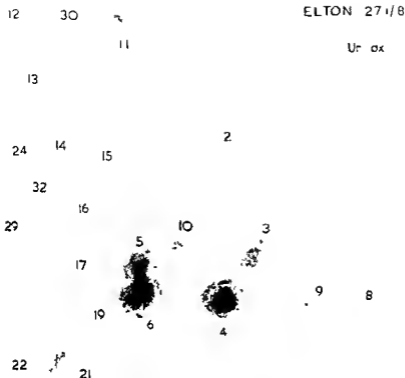


PLATE II. Normal urine: oxidized

An amount of urine equivalent to 250  $\mu$ g of nitrogen is pipetted onto the cross in the right-hand corner

Phenol is run in a horizontal direction, collidine and lutidine vertically

- |                             |                                   |
|-----------------------------|-----------------------------------|
| 1. Cystine as cysteic acid  | 14. Valine                        |
| 2. Taurine                  | 15. Methionine                    |
| 3. Serine                   | 16. $\alpha$ -amino-n-butyric     |
| 4. Glycine                  | 17. Histidine                     |
| 5. $\alpha$ -alanine        | 18. $\beta$ -amino-iso-butyric    |
| 6. Glutamine                | 19. $\beta$ -alanine-n-citrulline |
| 8. Aspartic                 | 21. Lysine                        |
| 9. Glutamic                 | 22. Arginine                      |
| 10. Threonine               | 24. Ethanolamine                  |
| 11. Tyrosine                | 29. Proline                       |
| 12. Phenylalanine           | 30. Tryptophane                   |
| 13. Leucine and iso-leucine | 32. Unknown                       |



+

**PLATE III Urine from a case of acute hepatic necrosis oxidized**

An amount of urine equivalent to 250  $\mu$ g of nitrogen is pipetted onto the cross in the right-hand corner

Phenol is run in a horizontal direction, collidine and butidine vertically

- |                            |                                   |
|----------------------------|-----------------------------------|
| 1 Cystine as cysteic acid  | 14 Valine                         |
| 2 Taurine                  | 15 Methionine                     |
| 3 Serine                   | 16 $\alpha$ -amino-n-butyric      |
| 4. Glycine                 | 17 Histidine                      |
| 5 $\alpha$ -alanine        | 18 $\beta$ -amino-iso-butyric     |
| 6 Glutamine                | 19. $\beta$ -alanine-n-citrulline |
| 8 Aspartic                 | 21. Lysine                        |
| 9 Glutamic                 | 22 Arginine                       |
| 10 Threonine               | 24 Ethanolamine                   |
| 11 Tyrosine                | 29. Proline                       |
| 12 Phenylalanine           | 30 Tryptophane                    |
| 13 Leucine and iso leucine | 32 Unknown                        |



In combination with estimations of alkaline phosphatase, the thymol flocculation is useful in the differential diagnosis of jaundice.

Phosphatase values  $>35$  units/100 ml. with negative or 1+ flocculations nearly always indicate obstruction.

### (3) ZINC TURBIDITY TEST

The turbidity produced by a buffered solution of zinc sulphate and serum is due to the  $\gamma$  globulin content and differs from the two foregoing tests in that other components in the serum are unnecessary. The divalent zinc ion forms an insoluble metal complex with  $\gamma$  globulin. The turbidities are usually measured in the same units as for the thymol turbidity.

The test becomes positive later than the cephalin cholesterol and the thymol turbidity tests, when abnormal values of  $\gamma$  globulin appear in the serum. It is usually the last test to become normal in recovery from viral hepatitis. It is nearly always negative in obstructive lesions but may be strongly positive in chronic liver diseases when the cephalin and thymol tests are normal and no jaundice is present.

It is often positive in a number of other diseases, such as lupus erythematosus, multiple myelomatosis, sarcoidosis and sub-acute bacterial endocarditis, where the serum  $\gamma$  globulin is often raised.

#### *Reagents :*

*Zinc Sulphate Buffer* ( $pH=7.5$ ). 24 mg. of  $ZnSO_4 \cdot 7H_2O$  together with 250 mg. of barbitone and 210 mg. of sodium barbitone are dissolved in 1 litre of water. The procedure and comparisons of turbidity with protein standards are the same as for the thymol turbidity test. Normal values are between 0 and 4 units. Values may exceed 10 units in chronic liver disease.

### (2) AMINO ACID METABOLISM

The liver maintains a balance between tissue and plasma amino acids and those derived from protein breakdown in the gut. In acute and chronic liver disease this function becomes disturbed. In acute liver disease, especially hepatic coma, there may be a general increase in concentration of all the amino acids in the body fluids and tissues. In the chronic forms, however, increases in concentration may be confined to a few amino

acids only. The amino acids spill over into the urine from the plasma when the reabsorptive capacity of the renal tubules for each particular acid is exceeded. The mechanism of the amino aciduria in liver disease differs from that in cystinuria and Fanconi's disease where specific renal tubular defects cause a failure of reabsorption of certain amino acids.

The normal level of plasma amino nitrogen is about 4.0 mg./100 ml., and rises to between 6.0 and 7.0 mg. after a heavy protein meal. In hepatic coma this figure may exceed 10 mg./100 ml.

The pioneering work by Dent of two-dimensional paper chromatography has enabled not only individual amino acids to be detected and studied in the various body fluids, but the recognition of certain well-defined and characteristic amino acid "patterns" in disease, especially those including the liver and kidneys.

### *Amino Acid Chromatography*

Specimens of urine, deproteinized cerebro-spinal fluid or plasma, are desalted in a special electrolytic apparatus. A known volume of liquid, usually 650  $\mu$ l. for plasma or cerebro-spinal fluid, and that equivalent to 250  $\mu$ g. of nitrogen for urine, is placed at one corner of a large (60 cm. x 60 cm.) square of filter paper, 6 cm. from each edge. One edge of the paper is allowed to dip into a trough of phenol saturated with water, the paper hanging vertically. The whole is enclosed in a glass tank fitted with an airtight lid. The phenol is allowed to travel down the paper almost to the edge. The paper is then removed and dried in a warm current of air. The process is repeated using a mixture of collidine and lutidine saturated with water, after turning the paper through 90°. After drying, the paper is sprayed with 0.1 per cent solution of ninhydrin in butyl alcohol, the amino acids appearing as blue spots after 24 hours and occupying individual and characteristic positions on the paper (see Plates II and III).

By this method the cerebro-spinal fluid and plasma show abnormal patterns in acute and chronic liver disease.

The urinary changes are the most striking. About 1.2 per cent of the daily protein intake in a normal person is excreted in the urine in the form of amino acids. Chromatography of

amounts of urine equivalent to 250  $\mu$ gm. of nitrogen show three normal patterns, with glycine alone, or glycine plus taurine, or glycine plus  $\beta$ -amino-isobutyric acid predominating. Small amounts of cystine, serine, glutamine and  $\alpha$ -alanine are also found. In hepatic coma due to infectious hepatitis the urinary pattern of amino acids shows that all the common ones are present in high concentration, with ethanolamine a substance not usually detected in the urine. In acute viral hepatitis, the pattern resembles that of hepatic coma in distribution, but the concentration of the amino acids is much weaker. Ethanolamine is usually present also. In chronic liver disease the pattern approaches that of the normal, but usually includes tyrosine, phenylalanine, and ethanolamine as well as increased amounts of taurine, cystine, and  $\beta$ -amino-isobutyric acid. When biliary obstruction is unaccompanied by parenchymal change, the urinary amino acid pattern is normal. Chromatography can provide a useful test in the differential diagnosis of jaundice.

### (3) BLOOD AMMONIA

In the last few years interest in blood ammonia levels in hepatic failure has been renewed, since these are often elevated.

Compared with the peripheral blood, the portal blood in the dog contains a much larger amount of ammonia. This is thought to be derived from bacterial breakdown of protein in the gut and under normal conditions is metabolized by the liver. Dogs with Eck fistulae (i.e. dogs in which an artificial connection has been made between the portal vein and the inferior vena cava, thus diverting the blood from the liver) can become drowsy and irrational in behaviour on being fed large amounts of protein. A parallel situation has been found in a number of patients with cirrhosis in whom collaterals between the portal and caval vessels have been demonstrated. Blood ammonia levels during these episodes have been found to be raised. High blood levels of ammonia in hepatic coma due to infectious hepatitis are explained by the failure of the liver to metabolize ammonia reaching it from the gut by way of the portal vein. Raised levels in chronic disease may be partly due to this cause and partly due to the extensive collateral circulation. So far, however, no close correlation has been shown to exist between blood ammonia

levels and the extent of the neurological and mental abnormalities.

The finding that glutamic acid, administered intravenously, can bring about a return to consciousness in a proportion of patients in hepatic coma, suggests that it might do so by removing the toxic ammonium ion by the formation of glutamine. Such a mechanism has been postulated for the normal removal of intra-cellular ammonia in the central nervous system.

Blood ammonia levels are conveniently estimated by the Conway micro-diffusion technique. A number of rigid precautions must be observed. Normal levels range from 2-10  $\mu\text{g.}/\text{ammonia nitrogen}/100 \text{ ml. of blood}$  to 30-100  $\mu\text{g.}$  in cirrhosis, while in hepatic coma values to 300  $\mu\text{g.}$  are often obtained.

#### (D) ALKALINE PHOSPHATASE

This enzyme is related to osteoblastic activity and blood levels are elevated in diseases of bone, especially hyperparathyroidism and Paget's disease. Hence these conditions must be excluded before interpreting changes in serum levels of the enzyme from the standpoint of liver or biliary disease. Selective staining of histological sections shows that the parenchymal cells of the liver normally contain little of the enzyme, but that the K  pffer cells and the cells lining the bile canaliculi show significant amounts. These cells may actually elaborate the enzyme since proliferation of the bile ducts is associated with high levels. On the other hand, the liver may contain an inactive form which becomes active only on excretion into the bile. The liver cells most probably excrete this enzyme rather than synthesize it. The means whereby this is accomplished is far from clear, and it seems to bear no relationship to the mechanism of excretion of bile pigments. When blood levels of bilirubin are high in the acute phase of hepatitis the alkaline phosphatase levels are usually only slightly raised, while in malignant obstruction of the biliary tree serum bilirubin may be slightly elevated only while the alkaline phosphatase values may reach 40-50 K.A. units/100 ml. of serum.

Thirty units per 100 ml. of serum is a useful figure in separating obstructive from non-obstructive lesions when taken into consideration with the flocculation tests.

Values greater than 30 units with negative or weak flocculation tests are indicative of pure obstruction, while levels less than this value with positive flocculation tests are almost certainly due to lesions involving the liver parenchyma. High levels of alkaline phosphatase with marked jaundice and strongly positive flocculation tests express severe parenchymal damage with marked intrahepatic obstruction.

Scrum choline esterase activity is also depressed in liver disease and has been recommended as a test to distinguish between obstructive and hepatogenous jaundice. The estimation is not an easy one to carry out and yields little information that cannot be obtained by the simpler liver function tests.

#### (E) CARBOHYDRATE METABOLISM

Carbohydrate metabolism can be grossly altered in hepatic disease. Abnormal glucose tolerance curves are often found in cirrhosis, while hypoglycaemia and increased blood pyruvate and  $\alpha$ -ketoglutarate are features of hepatic coma. Owing to the number of extrahepatic factors, glucose tolerance measurements are of little help in the diagnosis of hepatic damage. Laevulose, once in vogue, has now been found to be utilized outside of the liver.

Galactose is converted to glycogen in the liver and tolerance can be measured after oral or intravenous routes. Even with this sugar, differential diagnosis appears to be limited to the early extrahepatic obstruction, when curves are normal. In long-standing obstruction, acute and chronic parenchymal lesions, tolerance to galactose is abnormal and curves show no features characteristic of the type of lesion.

#### (F) FAT AND CHOLESTEROL METABOLISM

Absorption from the gut of fat is largely dependent upon the formation of stable emulsions of a critical particulate size and bile salts form an essential component of these emulsions. Complete absence or relative lack of these compounds in the alimentary tract due to intra- or extra-hepatic biliary obstruction, results in an increase of fat in the stools. Low plasma calcium values result, occasionally with tetany, in long-standing obstruction, due to faulty calcium uptake.

Blood cholesterol levels, although dependent on thyroid

activity and hereditary factors, are related to liver function. Cholesterol is synthesized, esterified, and degraded mainly in the liver and parenchymal damage results in a fall of both total cholesterol and the esterified fraction. Obstructive jaundice on the other hand gives increased values of both of these fractions, until resultant cellular damage when low values are reached. It is difficult to understand why high values should obtain in obstruction since the bile itself contains only a small proportion of cholesterol.

Plaques, containing cholesterol material, and called xanthomata, are occasionally observed on the inner canthi and elsewhere on the skin in cases of long standing biliary cirrhosis. They are always associated with high blood values. The chemical techniques for both the total and ester fraction are difficult to perform. The test is used more for research than for routine purposes.

#### (G) ELECTROLYTE METABOLISM AND HORMONE DESTRUCTION

Plasma sodium and potassium levels are often disturbed in liver disease, especially in the very acute and cirrhotic stages. When vomiting accompanies impending coma, sodium levels may fall considerably, although acidotic levels similar to those seen in diabetic coma are seldom found. Low levels of both sodium and potassium are found in cirrhotics with ascites and frequent removal of ascitic fluid tends to deplete the body of these ions to an even further extent. The sensitivity of patients in hepatic coma, whether of acute or chronic onset, to electrolytes and water may be due to the failure of the liver to destroy antidiuretic hormone and the mineralosteroids. Urinary 17-ketosteroid output is much reduced in chronic liver disease and administered cortisone fails to produce an increase.

Testicular atrophy, gynaecomastia, and loss of hair in the male, atrophy of the breasts and uterus, and menstrual disorders in the female may all result from abnormal hormone metabolism, in consequence of liver dysfunction. It is doubtful if the spider naevi and palmar erythema so characteristic of chronic liver disease are the result of high oestrogen blood levels.

From the foregoing account, a number of tests is available

for investigating and interpreting liver disease. Those that can be carried out with a minimum of labour and time are to be preferred, and some of the simplest tests give highly reproducible and reliable information. A large number of studies has concentrated on attempts to correlate the degree of hepatic dysfunction as revealed by the biochemical tests, with the extent of liver damage shown by histological examination of post-mortem or biopsy material. Although there is evidence of correlation existing between specific histological features and certain tests, at present it is impossible to correlate any one test with any given histological pattern of liver disease.

As stressed previously the liver function tests give the greatest help in diagnosis and prognosis when combined with an adequate history and clinical examination of the patient.

For routine use in suspected liver disease, the following tests should be used:

- (1) total serum bilirubin;
- (2) serum alkaline phosphatase;
- (3) cephalin flocculation, thymol, and zinc sulphate turbidity tests; and
- (4) qualitative urinary tests for bilirubin, urobilin and urobilinogen.

Raised serum bilirubin and the presence of urinary bile pigments establish the presence of jaundice.

When jaundice is present clinically, the determination of the serum bilirubin establishes a figure for its intensity and hence a baseline for comparison with future values while determination of the alkaline phosphatase and the flocculation tests serve to point to the etiology of the condition. Strongly positive flocculation tests together with alkaline phosphatase levels below 30 units/100 ml. suggest involvement of the liver parenchyma, while negative or weakly positive flocculation tests and phosphatase values exceeding 30 units are indicative of obstructive lesions.

#### THE DIFFERENTIAL DIAGNOSIS OF OBSTRUCTIVE JAUNDICE

Most cases of obstruction of the biliary tract by stones present no difficulty especially when a characteristic history of colic is obtained. Raised serum bilirubin with bilirubinuria are transient

features, while the flocculation tests, especially the cephalin, may become faintly positive if much infection is present.

Occasionally, however, obstruction due to stones and neoplasms may produce little constitutional disturbance and the clinical picture may then resemble sub-acute or chronic hepatitis. The liver may be palpable and tender in both conditions and an enlarged spleen and spider naevi should be looked for. Patients with chronic liver disease do not withstand laparotomy well while unrelieved obstruction leads eventually to damage of the liver parenchyma. Bilirubinuria may be present in varying degrees with jaundice in both conditions but urobilinogen is not found in the urine in obstruction in the absence of marked infection and secondary parenchymal damage. The alkaline phosphatase is raised above the value of 30 units/100 ml. of serum in the vast majority of cases of extrahepatic obstruction while the flocculation tests, positive in chronic liver disease, are normal. The thymol flocculation is almost never complete in obstructive lesions even when some secondary liver damage is present. Low serum albumin and high globulins, especially the  $\gamma$  fraction, characterize chronic hepatitis while the values remain normal in obstruction. Prothrombin levels are often low in both conditions, while chronic hepatitis alone fails to respond to parenterally administered vitamin K. Amino acid chromatography, normal in obstruction, shows increased excretion of cystine, tyrosine and phenylalanine along with several other amino acids in cases of chronic hepatitis. In equivocal cases liver biopsy or even laparotomy are often necessary.

When the obstruction is due to carcinoma of the head of the pancreas, jaundice, unaccompanied by urinary urobilinogen becomes severe, often with little constitutional disturbance beyond intractable itching of the skin. Severe boring epigastric pain radiating through to the back, a palpable gall bladder, and an accompanying glycosuria may focus attention on the pancreas. Alkaline phosphatase values may be extremely high while determination of faecal stercobilinogen often shows very low daily outputs of less than 5 mg. Since in acute hepatitis the values lie between 5 and 10 mg. daily, and in obstruction due to stones between 10 and 15 mg., the finding of such low amounts is almost diagnostic.

Very high alkaline phosphatase values above 40 units/100 ml. when bone lesions and parathyroid disease are excluded are characteristic of malignancy involving the biliary tract, while the absence of thymol flocculation in jaundice is highly suggestive of obstruction.

### HEPATIC COMA

This syndrome can result from liver insufficiency due to severe attacks of viral or homologous serum hepatitis, or more rarely from chloroform or phosphorus poisoning. It can occur also as a terminal event in chronic hepatitis and cirrhosis. In the former, drowsiness passes quickly to restlessness and mania followed by coma with signs of pyramidal involvement. The final stage is heralded by flaccid paralysis. In the more chronic conditions progress of the coma is slower, is characterized by a longer period of drowsiness and bizarre behaviour and is less likely to include episodes of mania.

Hepatic coma may supervene when the attack of viral hepatitis is still in the pre-icteric stage and the only positive findings then are a slightly raised serum bilirubin and urobilinogen in the urine. When occurring terminally in chronic disease, urobilinogen is nearly always present in the urine, often with bilirubin and hyperbilirubinaemia. Alkaline phosphatase is raised, albumin low, while  $\gamma$  globulin values are increased often to 3 and 4 g. per cent. All the flocculation tests are usually positive and frequently strongly so. In the acute type of coma the urinary amino acid pattern shows that practically all the common amino acids are excreted in great excess while ethanolamine is frequently seen. Plasma patterns also show increases of all the amino acids together with large amounts of methionine. The values of plasma  $\alpha$ -amino nitrogen are raised from 4.7 mg. per cent to 10 mg. or over. The CSF also shows an increase.

In the chronic type however, there is less disturbance in the body fluids of amino acid patterns. The plasma shows increased cystine and methionine while the urine may show minimal change from the normal or increases of cystine and ethanolamine together with small amounts of tyrosine, phenylalanine, leucine and valine.

In both types of coma the liver becomes smaller, foetor hepaticus is detectable in the breath, fluid retention with oedema occurs and widespread bruising becomes evident with haematuria, melaena, and epistaxis all failing to respond to vitamin K therapy. Hypoglycaemia may occur but is unusual with modern practice of intravenous therapy with solutions containing glucose.

Blood ammonia levels are usually raised in both types.

The prognosis of hepatic coma from all causes is poor. Recent experience with intravenous solutions containing sodium glutamate (Walshe 1953), has given encouragement in treatment.

When recovery from coma does take place in acute conditions, the surviving liver cells usually regenerate in a manner which damages the normal architecture to a degree that makes for the establishment of cirrhosis and eventual liver failure.

### CIRRHOSIS

A cirrhotic liver may result from a number of different disease processes including vascular congestion, biliary obstruction, inadequate nutrition (alcoholism) and viral hepatitis. For the sake of convenience cirrhosis implies here chronic hepatic disease resulting from either nutritional causes, vascular or biliary congestion, etc., while that arising from viral hepatitis is dealt with under a separate heading of "chronic viral hepatitis."

Liver function tests give no means of differentiating cirrhosis developing after acute conditions from that developing from nutritional (alcoholism, diabetes, ulcerative colitis, etc.) or other causes.

Cirrhosis is nearly always accompanied by vague ill health and fatigue after exertion and by loss of weight and by spider naevi over the upper part of the body and hepatosplenomegaly. Ascites and peripheral oedema are often present also. It frequently terminates in severe haemorrhage from oesophageal varices, hepatic coma or inter-current infection.

Urobilinogen is seldom absent from the urine while the urinary chromatogram usually shows increased excretion of cystine, taurine, and ethanolamine along with a smaller amount of tyrosine and phenylalanine. Serum bilirubin may be slightly raised depending on the extent of intra-hepatic obstruction to

the biliary outflow. Serum albumin is nearly always low, values less than 2 gm./100 ml. indicating a very poor prognosis, while the  $\gamma$  globulin fraction is always elevated to some extent. The flocculation tests are often strongly positive while the alkaline phosphatase may reach levels seen in purely obstructive lesions. Prothrombin is lowered and fails to return to normal values with vitamin K. When accompanied by ascites and oedema, plasma sodium and potassium are low.

### VIRAL HEPATITIS

It is thought that two viruses can give rise to a common clinical picture of hepatitis. Viral hepatitis spread by faecal or droplet contamination has an incubation period differing from that of homologous serum hepatitis which is spread by inoculation only. Homologous serum hepatitis appears to produce more sequelae while there is no cross immunity between the two types. The widespread epidemics of viral hepatitis during the war years among military personnel has led to a greater understanding of the disease and its prognosis. Fig. 13 is a scheme depicting the clinical course. The duration and severity vary widely.

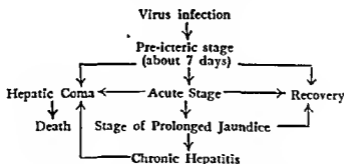


FIG. 13. Diagrammatic representation of clinical course of viral hepatitis.

#### (1) PRE-JAUNDICE STAGE

The patient may present with a vague illness and recovery within a few days. Serum bilirubin is seldom raised but the Van den Bergh reaction is direct. Bilirubin and urobilinogen appear in the urine but apart from the cephalin test, which is usually 1+ - 2+, the flocculation tests and alkaline phosphatase are normal. Very occasionally with an overwhelming infection this

stage passes into hepatic coma but the commonest course is to pass into the acute stage.

## (2) THE ACUTE STAGE

Occasionally this is heralded by severe upper abdominal pain simulating surgical conditions before the jaundice becomes noticeable. More commonly the vague pre-jaundice stage passes into frank jaundice, anorexia, nausea and pyrexia, with lymphocytosis and tenderness over the liver area. There is hyperbilirubinaemia with a direct Van den Bergh reaction, bilirubin, urobilin and urobilinogen with bile salts in the urine while the alkaline phosphatase rises as the jaundice increases. This stage usually lasts several weeks and generally during this time the stools become very pale and urobilinogen and urobilin disappear from the urine, indicating that practically no bile is reaching the alimentary tract. Clinical improvement is foreshadowed by the return of colour to the stools and the reappearance of urobilin and urobilinogen in the urine so that daily examination of these is a useful guide to progress. The cephalin test becomes positive much earlier than do the zinc sulphate and thymol turbidity. Plasma albumin and prothrombin fall slightly after a few weeks and  $\gamma$  globulin tends to rise. All these liver function tests continue to be positive for some time after clinical recovery has taken place. This is especially true of the flocculation tests and  $\gamma$  globulin which may remain abnormal for a year after the initial attack without relapse and thus they do not afford much help in assessing progress in the absence of clinical features. The role of too early a return to full activity in producing a state of chronic hepatitis after an attack is not clear. Most people agree that to be on the safe side providing the patient is otherwise well, a non-tender liver, serum bilirubin below 2 mg./100 ml. and bromsulphthalein retention of less than 10 per cent in 45 minutes, are good indications for convalescence. Serial bromsulphthalein retention tests with zinc turbidity estimations are most useful in follow-up in doubtful cases of complete clinical recovery.

## (3) STAGE OF PROLONGED JAUNDICE

In a few of the acute cases the jaundice remains intense for

weeks with absent urinary urobilin and urobilinogen and pale fatty stools. Serum alkaline phosphatase may reach much higher levels than those obtaining in the acute stage while the falls of albumin and prothrombin are more marked; the flocculation tests are more abnormal while the  $\gamma$ globulin values become higher. These cases generally take longer to resolve and a proportion of them drift into the chronic stage. Convalescence is often prolonged and adequate follow-up is essential.

### CHRONIC VIRAL HEPATITIS

A number of patients going through the prolonged jaundice stage suffer repeated relapses with ill health, tender liver and jaundice. In this group also are cases which give no history of an attack of hepatitis, who are seen as the result of frequent bouts of illness accompanied by jaundice. There is often a marked degree of hepatosplenomegaly. The liver function tests fluctuate with the clinical course but usually settle to become grossly abnormal. Bilirubinaemia is often high and alkaline phosphatase reaches levels typical of obstruction due to carcinoma. The albumin level is often low while the  $\gamma$ globulin fraction rises to very abnormal levels. Urinary amino acid patterns resemble those seen in cirrhosis. Portal collaterals become established and death may result from haemorrhage or hepatic coma. These cases may eventually settle down with much scarring of the liver.

### HAEMOLYTIC JAUNDICE

When this is uncomplicated by stone formation and obstruction, serum bilirubin values are raised, the Van den Bergh reaction being mainly indirect, while the urine contains both urobilin and urobilinogen but no bilirubin. The faeces are often heavily pigmented and determination of the daily stercobilinogen output is often in excess of the normal 200 mg. The rest of the liver function tests are normal.

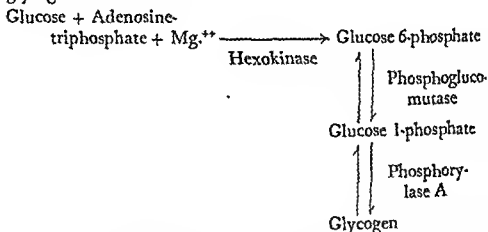
In familial acholuric jaundice and similar cases where rapid breakdown of haemoglobin produces pigment stones and intermittent obstruction of the biliary tree, serum alkaline phosphatase is often raised while bilirubin and bile salts accompany urobilinogen in the urine. The Van den Bergh test shows both the direct and indirect reaction.

in the liver. Galactose then appears in the urine where it must not only be distinguished from glucose but must be identified, as the continued administration of galactose as milk sugar (lactose) will lead to serious consequences.

## (2) THE LIVER

The liver is normally capable of phosphorylating all monosaccharides and thus directing their metabolism through the glucose pathway. In the metabolic defect leading to galactose intolerance, the enzyme galactowaldenase is congenitally absent and thus galactose 1-phosphate cannot be converted to glucose 6-phosphate.

A portion of the glucose reaching the liver is converted to glycogen as follows:



The reverse process is brought into play if the blood sugar falls below normal, glycogen being broken down by phosphorylase to glucose 1-phosphate which is then converted to glucose 6-phosphate, which is hydrolysed by a specific glucose 6-phosphatase, present only in liver, to glucose. The specific enzyme is required because the hexokinase reaction is virtually irreversible. It has recently been shown by G. T. Cori that glycogen storage disease in at least one of its forms is due to the absence of this enzyme in the patient.

The process of glycogen breakdown is greatly favoured by two hormones, adrenaline which acts in both liver and muscle, and glucagon which acts only in liver. Both hormones have been shown by Sutherland and De Duve to convert the relatively inactive Phosphorylase B to the active form, i.e. Phosphorylase A.



(a)

(b)

(c)

PLATE IV. Benedict's test for glucose in urine

(a) Benedict's test with three per cent glucose

(b) Benedict's test with one per cent glucose

(c) Benedict's solution

NOTE—For purposes of reproduction, three times the normal quantity of reagent and urine was used



However, not all the glucose arriving in the liver is converted to glycogen. Portion passes through and is utilized in all other tissues, notably muscle and brain, the latter organ being virtually unable to utilize any other substrate for its energy requirements. Also part of the glucose phosphorylated in the liver is oxidized or utilized for the synthesis of fats, proteins and other body constituents.

The glucose which passes to other tissues is utilized in a similar manner but these tissues are not capable of converting their carbohydrate stores back to glucose; e.g., muscle, under the influence of adrenaline, converts its glycogen to lactic acid which then passes to the liver where it is converted to glycogen and thus becomes available as glucose.

One of the most important functions of liver is to form glucose from non-carbohydrate sources (neoglucogenesis). This process consists of the conversion of the keto-acid residues of certain amino acids (glycine, alanine, serine, cystine, aspartic acid, glutamic acid, ornithine, proline, arginine), and the glycerol residue of fat, to glucose. Neoglucogenesis is most important in the maintenance of the blood sugar level under conditions of fasting. It is greatly enhanced by the action of the glucogenic steroids of the adrenal cortex (17 hydroxy-corticosterone), and is antagonized by the action of insulin.

### (3) ENDOCRINE GLANDS

It may be said that the level of glucose in the blood is a function of the endocrine balance of the body.

#### (i) *The Islets of Langerhans*

The islets of Langerhans consist of two types of cell, the Alpha cells and the Beta cells. The Beta cells produce the hormone insulin, the functions of which may be summarized as follows:

- (a) Acceleration of the utilization of glucose by all tissues with the exception of brain and red blood corpuscles.
- (b) Acceleration of the deposition of glycogen in all such tissues, but especially in liver and muscle.
- (c) As a secondary phenomenon the acceleration of the synthesis of fats and proteins.
- (d) The inhibition of neoglucogenesis. It is doubtful whether

this is, a primary phenomenon, or whether it is due to the more general effect favouring protein synthesis from amino acids already present in the tissues.

The Alpha cells appear to produce a hormone (glucagon) which aids in the breakdown of liver glycogen to glucose. Whether it has any other functions antagonizing the effect of insulin is doubtful at the present time.

Generally speaking, the action of insulin is opposed by the anterior pituitary, the adrenal cortex, the Alpha cells, and by adrenaline from the adrenal medulla.

## (ii) *The Pituitary Gland*

(a) *The anterior lobe.* It was shown originally by Houssay, and since confirmed by other workers, that the anterior lobe of the pituitary produced a substance which antagonized the effect of insulin. The evidence is based on the fact that removal of the anterior lobe produces insulin hypersensitivity, and also on the fact that removal of the anterior lobe in the diabetic animal restores the blood glucose level to within the normal range. More recently Young and his co-workers have shown that injection of anterior pituitary extracts produces diabetes in the intact adult cat and dog, and eventually leads to the destruction of the Beta cells of the pancreas.

Further important evidence in respect to this problem was obtained by many workers; initially, Corkill and Nelson utilized isolated tissues, usually rat diaphragm or liver slices in controlled in vitro systems. All workers in the field are agreed that the utilization of glucose and all synthetic phenomena are depressed by the injection of anterior pituitary extracts prior to the removal of the tissue, and that this inhibition is reversed by the action of insulin; and also that a similar depression is observed in tissues taken from diabetic animals, and that this depression can be reversed by insulin, or by the removal of the pituitary or the adrenals.

The anterior pituitary hormone responsible for these effects has been identified with crystalline-growth hormone. Although it may appear contradictory that the phenomenon of growth is associated with a diabetogenic effect, all attempts to separate the two have so far been unsuccessful.

Many actions have been ascribed to growth hormone, but the two most important in diabetes are, first and foremost, that it appears to be a direct antagonist of insulin at the site of action and secondly, that it is a trophic factor for the Alpha cells of the pancreas, thus leading to the secretion of glucagon. It is, of course, possible that the two effects mentioned are related in action.

(b) *The posterior lobe.* Although crude extracts of the posterior lobe have been shown to be able to raise the blood sugar during insulin hypoglycaemia, the pure hormones have no effect on carbohydrate metabolism and this action must be regarded as being due to contamination by anterior pituitary hormones.

### (iii) *Adrenal Glands*

(a) *The adrenal cortex.* The glucogenic hormone of the adrenal cortex (Compound F) (17 hydroxy-corticosterone) acts in two ways to antagonize the action of insulin. It is a direct antagonist at the site of action of insulin, acting in conjunction with the pituitary, and secondly, it stimulates neoglucogenesis by favouring the breakdown of tissue proteins. However, it has one action which is synergistic with insulin, and that is to favour the deposition of liver glycogen. The proof of the antagonism is based on the work of Long, who showed that experimental diabetes was ameliorated by adrenalectomy and that the diabetic state could be restored by injection of adrenal cortical extracts but not by the injection of adrenaline.

(b) *The adrenal medulla.* The action of adrenaline in mobilizing liver and muscle glycogen has already been discussed.

### (iv) *The Thyroid*

The role of the thyroid in carbohydrate metabolism is not well defined, although many cases of thyrotoxicosis show glucose in the urine. At present it appears that the role of the thyroid is to speed up metabolism generally, and that carbohydrate metabolism follows the general pattern of the organism.

### (v) *The Gonads*

Recently some evidence has been presented which tends to indicate that the oestrogens are anti-diabetogenic, whereas the

androgens tend to be diabetogenic in their action on carbohydrate metabolism.

#### (4) NERVOUS STIMULI

Nervous stimuli tend to break down liver glycogen, the action being possibly mediated by adrenaline.

#### (5) THE RENAL THRESHOLD

Although the renal threshold is normally about 180 mg./100 ml., in some persons it may fall as low as 120 mg./100 ml. or even lower, leading to a persistent glycosuria, although the patient remains in good health. This state is differentiated by blood sugar estimations, done in conjunction with urinary glucose tests, and it is seen that glucose is present in the urine at normal blood glucose levels.

### TYPES OF GLYCOSURIA

#### (1) DIABETIC GLYCOSURIA

Removal of the pancreas in man or in laboratory animals leads to an almost immediate hyperglycaemia, glycosuria, and disturbances of fat and protein metabolism. That this is due to removal of the Beta cells of the islets is shown by the fact that ligation of the pancreatic duct which leads to the atrophy of the acinar tissue does not produce diabetes, and further that the injection of a suitable dose of alloxan monohydrate produces a diabetic state. Alloxan, when correctly employed, specifically destroys the Beta cells.

However, pathological examination of the islets of diabetic patients has shown that in many of these the islets are normal. Thus, together with the demonstration of specific antagonists of insulin, the possibility arises that in some cases the diabetes may not be due to a failure to secrete insulin, but to an overaction of antagonists leading to a relative lack of insulin. This view has recently had experimental support from the work of Wrenshall, who showed that insulin was present in the pancreas of nearly all diabetics with an age of onset of over 20, and that of Bornstein who detected insulin in the peripheral blood of some diabetics. It must be emphasized, however, that in all cases of diabetes mellitus a lack of insulin exists. This may be due to

failure of secretion or metabolic demands beyond the secretory capacity.

In diabetes mellitus due to either the absolute or relative lack of insulin, two factors combine to raise the blood glucose. The first and main factor is the failure to utilize glucose, and the second is the effect of uncontrolled neoglucogenesis, although this plays a relatively minor role in the elevation of the blood glucose level. As such a hyperglycaemia invariably exceeds the renal threshold, glucose spills over into the urine and is easily detected.

In the disease known as haemochromatosis, there is a disturbance of iron metabolism, leading to the deposition of the iron containing pigment haemosiderin, in the liver, spleen, pancreas, lymph glands and skin. The deposits in the pancreas lead to fibrosis and destruction of the islets and hence to a diabetic state due to the lack of insulin. This condition is known as bronze diabetes.

## (2) GLYCOSURIA OF ENDOCRINE DISORDERS, OTHER THAN DIABETES

Diabetes mellitus is the classical example of an endocrine glycosuria, but glycosuria occurs in endocrine disorders other than diabetes.

(a) *Thyrotoxicosis*. Glycosuria has been observed in about 30 per cent of cases of thyrotoxicosis. However studies of blood sugar levels and glucose tolerances have shown that this glycosuria is purely symptomatic and not indicative of any impairment of ability to utilize carbohydrate. In a large group of patients with thyrotoxicosis who were exhaustively investigated only 1½ per cent had true diabetes mellitus, which proportion is very little above that for the population as a whole.

(b) *Cushing's Syndrome (bilateral adrenal hyperplasia)*. In this condition, there is a great hypersecretion of adrenal glucocorticoids leading to insulin resistance and a diabetic-like state which is corrected by removal or partial removal of the adrenal glands.

(c) *Acromegaly (secreting tumour of the acidophil cells of the anterior pituitary)*. In this growth, glycosuria is frequently present and often assumes the proportions of severe diabetes mellitus. This is thought to be due to hypersecretion of growth hormone.

(d) *Pheochromocytoma* (secreting tumour of the adrenal medulla). In this condition, the main symptoms are of an intermittent severe hypertension due to hypersecretion of nor-adrenaline, but such tumours also hypersecrete adrenaline, and give rise to glycosuria by causing a rapid breakdown of liver glycogen.

### (3) RENAL GLYCOSURIA

As previously mentioned, the renal threshold for glucose may be considerably lowered in some subjects, so that glycosuria occurs in the presence of a normal blood sugar. This condition is differentiated from diabetic glycosuria by the study of the relation of blood glucose levels to the appearance of glucose in the urine (Glucose Tolerance Test).

Phloridzin produces a similar state, apparently by inhibiting the enzyme system concerned with the reabsorption of glucose in the proximal tubule.

Renal glycosuria cannot be altered by therapy, but appears to be harmless to the individual.

### (4) NERVOUS GLYCOSURIA

This condition is frequently seen in recruits for military service and during insurance examinations and thus is of considerable importance. It is differentiated by the study of the blood glucose level, and by the fact that it is intermittent and only appears when mental stress is present.

Puncture of the floor of the fourth ventricle in some laboratory animals may cause hyperglycaemia and glycosuria, due to impulses passing down the spinal cord and along the splanchnic nerves (a) to the supra-renal glands, liberating adrenaline, which induces hyperglycaemia, and (b) to the liver, where a rapid hydrolysis of stored glycogen occurs (glycogenolysis).

The autonomic nervous system plays an important part in the regulation of the secretion of the endocrine glands, and its excessive stimulation, as in fright or in accidents causing injury to the base of the skull, may be a factor in the development of glycosuria in man.

In a recent communication of Corkill and Marks emphasis has again been placed on the importance of the emotional

factor in provoking glycosuria. This is particularly noticeable in the examination of recruits for the army.

Furthermore, a subject showing symptomless glycosuria should not be classified "diabetic" as a result of a single glucose tolerance test.

### (5) HEPATIC GLYCOSURIA

It is difficult to determine whether a disorder of liver function ever produces glycosuria, unless other metabolic deficiency is present, but a raised blood sugar is at least theoretically possible under some conditions due to the excess breakdown of glycogen, as may be seen from Fig. 14.

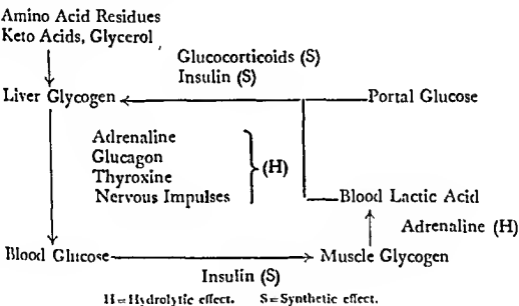


FIG. 14

### (6) ASPHYXIAL GLYCOSURIA

Glycosuria is frequently observed during asphyxia and is apparently due to lack of oxygen. This may be part of a stress phenomenon, and due to the action of adrenaline and adrenal glucocorticoids, although a direct action due to lack of oxygen cannot be excluded. In anoxaemia of ordinary severity there is stimulation of the central nervous system and the splanchnic nerves convey impulses directly to the liver cells, causing glycogenolysis, or to the supra-renal glands, causing an increased

amount of adrenaline to be liberated, which, in its turn, causes hyperglycaemia. Severe anoxaemia, however, can act directly on both liver cells and supra-renal glands, with the production of hyperglycaemia and glycosuria.

Some factors influencing the percentage of sugar in the blood are shown in Fig. 15.

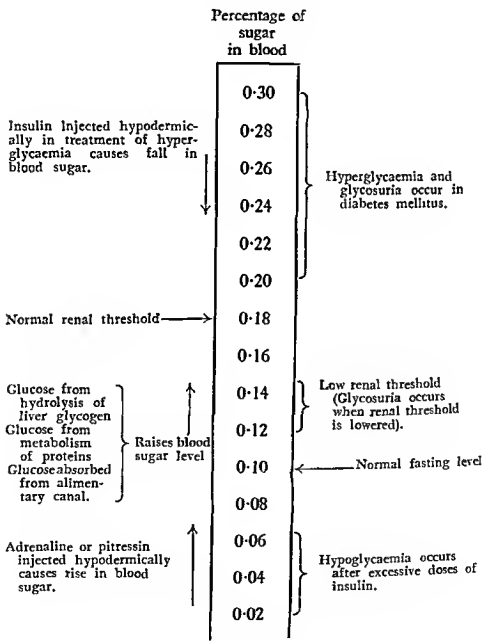


FIG. 15. Some factors influencing the percentage of sugar in the blood

## (7) ALIMENTARY GLYCOSURIA

Although a condition of glycosuria has been described following the ingestion of large amounts of glucose, it is extremely doubtful whether it exists in any normal person. Probably most cases described have had minor defects of carbohydrate metabolism.

## QUALITATIVE TESTS FOR GLUCOSE IN URINE

## (1) BENEDICT'S TEST

*Principle.*—Benedict's qualitative sugar solution contains sodium carbonate, which is a much weaker alkali and has less tendency to destroy small quantities of glucose than has the sodium hydroxide contained in Fehling's solution. In addition, it does not decompose urea (as does NaOH) with the liberation of ammonia, which may hold the cuprous oxide in solution. The principle of the test depends upon the aldehyde reduction of a copper salt in an alkaline medium (see Plate IV).

*Reagent.*—Benedict's qualitative glucose reagent (p. 356).

*Procedure.*—To 5 ml. of Benedict's reagent in a test tube add 8 drops of urine. Boil the mixture vigorously for two minutes, or heat on a boiling water bath for five minutes, and then allow the fluid to cool spontaneously. In the presence of glucose the entire body of the solution will be filled with precipitate, which may be red, yellow or green in colour depending upon the amount of sugar present. If no glucose be present, the solution will remain perfectly clear. Adhering rigidly to the above technique, uric acid and urates do not reduce Benedict's solution, but other reducing substances must be excluded before the reduction can be considered as due to glucose.

There is evidence that Benedict's test frequently shows a slight reduction due to glucose even when the urine tested is that of a normal individual.

(a) *Fructose.*—Fructosuria is a rare condition. It may occur after the ingestion of large amounts of fruit or honey, this being called alimentary fructosuria. Essential fructosuria also occasionally occurs. It is a metabolic error, in which there seems to be a failure of the liver to convert fructose to glycogen and this laevo-rotatory monosaccharide appears in the urine. Two groups

of patients are recognized—(a) those in which both glucose and fructose are excreted and (b) those in which fructose is alone excreted. Fructosuria seems to be a harmless condition. Fructose can only be satisfactorily identified by either paper chromatography or polarimetry.

(b) *Lactose*.—This may occur in the urine of women during lactation, and it readily reduces Benedict's solution. Any lactose reabsorbed into the circulation from the mammary gland cannot be utilized by the body and is therefore excreted in the urine. It is distinguished by its characteristic osazone, which has the appearance of wattle blossom, and by the fact that it does not ferment with yeast. It is difficult to prepare an osazone from a urine containing less than 0.5 per cent lactose. A glucose tolerance test with associated blood analyses will decide whether there is an accompanying hyperglycaemia and diabetes mellitus. Lactosuria has no pathological significance.

(c) *Galactose*.—As previously mentioned, galactose may appear in the urine of children with an inborn error of metabolism. Such children apparently are born with an inability to convert galactose to glucose in the liver, and hence galactose appears in the urine. As the condition is serious, it is necessary to identify the sugar. This is done most satisfactorily by paper chromatography. Galactose is also not fermented by yeast.

(d) *Pentose*.—After the excessive ingestion of cherries, prunes and certain other fruits, pentoses may appear in the urine (transient or alimentary pentosuria). Pentosuria also occurs as a result of an anomaly of carbohydrate metabolism (essential pentosuria) and such pentosuria may be familial and is most frequently found in those of the Jewish race. The pentose in urine is either arabinose or xyloketose, the former being present usually as an optically inactive mixture and the latter, which is more commonly present, being dextro-rotatory. Fischer and Reiner found that pentose tolerance curves (blood sugar) were the same in pentosuric as in normal children. These curves differed from the normal dextrose tolerance curves, in that the rise came more slowly and was longer sustained. The ingestion of amidopyrin, in particular, and also of codein and luminal increases the output of urinary pentose. Drugs which are excreted in the urine as compound glycuronates tend to increase the urinary pentose.

Pentoses cause the reduction of Benedict's solution. They may be distinguished by the fact that they do not ferment with yeast, and also by the following tests:—

### 1. Tollen's Test.

*Procedure.*—To 3 ml. of urine add an equal volume of strong hydrochloric acid and a piece of phloroglucin about the size of a pea and heat the mixture on a water bath at 100° C. A cherry-red colour develops and the solution shows an absorption band between D and E. On cooling, a dark precipitate separates out.

### 2. Bial's Orcin Test

*Procedure.*—To about 3 ml. of urine add 5 ml. of Bial's reagent (p. 357) and heat to boiling point. A green colour or the formation of a green precipitate indicates pentoses. The solution shows two absorption bands, one in the red between B and C and the other near D line.

### 3. Aniline Acetate Test

*Procedure.*—To 3 ml. of urine add an equal volume of strong hydrochloric acid and boil. Hold over the mouth of the test tube some filter paper soaked in aniline acetate. The development of a pink colour indicates the presence of pentose in the urine.

TABLE I

The Urine in Pentosuria, Renal Diabetes and Diabetes Mellitus

	<i>Pentosuria</i>	<i>Renal Diabetes</i>	<i>Mild Diabetes Mellitus</i>
Reducing bodies in urine—			
(a) Fasting	+	+	0
(b) Two hours after carbohydrate meal	+	++	+++
Fermentation of urine	Negative	Positive	Positive
Bial's test	Positive	Negative	Negative
Tollen's test	Positive	Negative	Negative
Urinary osazone—			
(a) Form	Needles	Sheaves	Sheaves
(b) Melting point	160°	204°	204°
Glucose tolerance test	Normal	Normal	Diabetic

(Modified from Enklewitz and Lasker)

(e) *Glycuronic Acid*.—This substance, so closely related to glucose in its chemical composition, may appear in the urine combined with putrefactive bodies, such as indoxyl and skatoxyl, or with drugs which have been administered, e.g., chloral, camphor, chloroform, phenazone, acetanilid, morphine, salol, sodium salicylate or benzoic acid. These conjugated glycuronates are harmless and in this combination toxic substances may be excreted.

Free glycuronic acid is dextro-rotatory, but almost without exception its combinations are laevo-rotatory. When it is excreted in the urine it is invariably in the conjugated form, hence urine which contains it is laevo-rotatory, unless glucose or some other dextro-rotatory substance is present. When heated with Benedict's solution, some, but not all, of the varieties of glycuronates are decomposed, yielding free glycuronic acid, which contains an aldehyde group and readily reduces Benedict's solution. The difference in reducing power of glycuronates is illustrated on the one hand by trichlorethyl-glycuronic acid which appears in urine after the administration of large doses of chloral hydrate, and readily reduces Benedict's solution, when boiled with it for a few moments; whilst on the other hand indoxyl-glycuronic acid, which may be increased in the urine in excessive intestinal putrefaction, shows no reducing power, even on prolonged boiling with Benedict's solution. Glycuronic acid and glycuronates do not ferment with yeast and so can be distinguished from glucose.

(f) *Alkapton (Homogentisic acid)*.—This substance is derived from the aromatic compounds—tyrosine and pbenylalanine—of protein and may represent a stage in their katabolism in the body. The urine containing alkapton is usually straw-coloured and rapidly darkens owing to oxidation of the alkapton, which becomes greenish-brown or even dark brown or black. These changes are greatly facilitated by the addition of a few drops of ammonia or potassium hydroxide to the urine. Alkapton reduces Benedict's solution, but does not ferment with yeast and gives no osazone. If ferric chloride solution be allowed to fall drop by drop into the urine, a momentary deep blue colour appears. Alkaptonuria is a rare condition which is usually congenital.

TABLE II

## Chief Reactions of Reducing Substances Found in Urine

Substance Reducing	Benedict's test	Osazone test	Bial's test	Fermentation test	Rotation of polarized light
Alkapton	+	—	—	—	Nil
Glycuronic acid	+	+	—	—	Conjugated form laevo-rotatory Free form dextro-rotatory
Pentose	+	+	+	—	Usually dextro-rotatory
Lactose	+	+	—	—	Dextro-rotatory
Fructose	+	+	—	+	Laevo-rotatory
Glucose	+	+	—	+	Dextro-rotatory

(+) Indicates reaction. (—) Indicates no reaction.

## (2) PHENYLHYDRAZINE TEST

*Principle.*—Reducing sugars when heated with phenylhydrazine form an osazone, which may be precipitated from solution as crystals having a characteristic appearance under the microscope.

*Reagents.*—Phenylhydrazine hydrochloride.  
Sodium acetate.

*Procedure.*—Introduce into a test tube a small quantity of phenylhydrazine and double its volume of sodium acetate. Half fill the test tube with urine, add a few drops of glacial acetic acid and mix thoroughly by inverting the test tube several times. Then place in a water bath at 100° C. for 20 minutes. Now remove the tube from the water bath and stand aside to allow the precipitate (if present) to settle. If glucose be present in pathological quantity, a yellow crystalline precipitate of glucosazone will occur, which, when examined microscopically, will be seen to have the characteristic form of delicate sheaves. The melting point of these sheaves is 204° C.

The only possible fallacy is the presence of glycuronic acid, the osazone of which is similar to glucosazone, but glycuronic acid does not ferment with yeast.

## (3) FERMENTATION TEST

*Principle.*—Yeast ferments glucose with the production of alcohol and carbon dioxide, and the latter, which can be readily

collected, serves as an index of the presence of glucose in urine.

*Reagents.*—1. Glucose solution (1 per cent).

2. Yeast.

*Procedure.*—Take three test tubes fitted with corks each having a small nick cut along the side. Half-fill the tubes with yeast suspension. Then fill them with the following solutions:

1. Urine under investigation, boiled to dislodge any carbon dioxide and then cooled.

2. Glucose solution.

3. Urine (containing no sugar), boiled and cooled.

Now tightly cork the tubes and invert them in a water bath at 40° C., where they are kept for one hour. If carbon dioxide is evolved, it accumulates at the top of the inverted tube and the displaced water escapes through the nick in the cork. If gas bubbles are seen to rise in appreciable number, in test tube No. 1, it is safe to assume that a fermentable sugar is present. The object of the control test (2) is to prove that the yeast is active, and control test (3) is to determine whether any gas is given off by the yeast itself.

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In a doubtful case of glycosuria it is necessary to try more than one of the above tests—Benedict's, the phenylhydrazine test, and the fermentation test. If a urine giving Benedict's reaction shows fermentation with yeast and yields a glucosazone, then glucose or fructose must be present. This latter sugar very rarely occurs in urine and is laevo-rotatory.

*Clinical value of qualitative tests for glucose in urine.*—Concerning the presence of glycosuria in the normal population, Blotner gives an incidence of glycosuria in 8 per 1,000 and 4.6 cases of diabetes per 1,000 in 45,650 selectees for the U.S. Army in the recent war. Weiden, working at the Women's Hospital, Melbourne, found that Fehling's solution was reduced by 4.4 per cent of urines of normal non-pregnant women but 47.7 per cent of the same urines gave some reduction if Benedict's solution were used. To determine the minimal amount of glucose which would show reduction of these solutions, Weiden dissolved glucose in urine which failed to reduce Benedict's solution. Fehling's test showed a slight reduction with a concentra-

tion of 0.12 per cent glucose which is in agreement with Benedict's statement that Fehling's solution detected the presence of glucose only when it was in excess of 0.1 per cent. Benedict's solution was slightly reduced in a concentration of 0.06 per cent glucose in urine. Moritz gives the mean value for reducing sugar in normal urine as 0.05 per cent. It would therefore seem that the high incidence of glycosuria found by Weiden when using the Benedict test is probably due to the inclusion of many urines with no more than the normal amount of glucose present. Using, then, some reduction of Fehling's solution as essential for deciding that glycosuria exists and confirming the presence of a reducing substance by use of the Benedict test and when possible the formation of glucosazone, Weiden showed that glycosuria occurred in 4.4 per cent of normal non-pregnant women, 29.6 per cent of women during the early months of pregnancy, and 19.8 per cent from about the sixth month to term. Glycosuria in the early months of pregnancy persisted to the end of pregnancy in most cases of these patients, but cleared up in the later months in some of them.

If a very slight reduction only is found on boiling the urine with Benedict's solution the following simple procedure may be adopted. The patient is given no food for 12 hours, e.g., he comes fasting in the morning and after completely emptying his bladder is given by mouth 50 grams of glucose dissolved in 150 c.c. of water. At the end of two hours he again micturates and the urine is tested for sugar. If there is no reduction or still only the slight reduction then the patient is not suffering from diabetes mellitus and there is no need to do a glucose tolerance test so that the bio-chemist is saved much unnecessary work.

If, after the procedure just described, glucose of clinical significance appears in the urine it is essential that the glucose tolerance test with blood sugar estimations, as described in Chapter V, should be performed to determine the intensity of diabetes mellitus and to exclude renal diabetes.

It cannot be too strongly emphasized that the diagnosis of diabetes mellitus should not be made merely on the detection of the presence of a reducing sugar in the urine.

*Quantitative Estimation of glucose in urine.* — Several methods are available for the quantitative estimation of glucose

in urine, but as these are rarely done in clinical practice, the student is referred to specific texts on the subject.

#### (4) PAPER CHROMATOGRAPHY

The introduction of paper chromatography into clinical biochemistry has given an entirely new tool for the qualitative identification of sugars in the urine. This method gives the most satisfactory identification of any sugar found in the urine. For practical details the student is referred to detailed texts on the subject.

TABLE III

Rf Values\* of various Common Sugars in several Solvent Systems

Sugar	Solvent 1	Solvent 2	Solvent 3
d Glucose	0.39	0.39	0.18
d Galactose	0.44	0.34	0.16
d Fructose	0.51	0.42	0.23
Lactose	0.38	0.24	0.09
d Xylose	0.44	0.50	0.28
d Arabinose	0.54	0.43	0.21

Solvent 1. Phenol-Ammonia (%W/V) with HCN.

Solvent 2. Collidine-water.

Solvent 3. n-Butanol-Acetic Acid-Water. (40:10:50 v/v).

These results were obtained in the Biochemistry Dept. Melbourne University and do not differ in any way from those obtained elsewhere.

\* Rf value represents the ratio between the distance moved by the compound and that movement of the mobile phase of the solvent.

Consideration of the table shows that identification may readily be made by using all three systems with reference compounds, and that in most cases two systems are quite adequate.

#### (5) POLARIMETRY

Polarimetry may be used for the identification and even estimation of reducing sugars in the urine, but unlike chromatography requires specialized equipment. The following table shows the specific rotations of certain common sugars.

TABLE IV

Specific Rotation of various Carbohydrates (a)<sup>d</sup>

d Glucose	+ 52.5	d Xylose	+ 19.0
d Fructose	- 93.8	Sucrose	+ 66.5
d Galactose	+ 81.5	Lactose	+ 52.5
d Mannose	+ 14.2	Maltose	+ 138.0
l Arabinose	+ 104.5		

A consideration of the table shows that although completely satisfactory for most sugars this technique will not differentiate glucose from lactose.

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## V

### PANCREATIC EFFICIENCY

#### FUNCTIONS OF THE PANCREAS

The functions of the pancreas are of a twofold character :

- (a) The production of two internal secretions concerned with carbohydrate metabolism: insulin, glucagon.

Although one known function of glucagon is to raise the blood sugar in hypoglycaemia, too little is known of its physiological functions for any practical methods for determining its activity in the patient to have been devised, and accordingly only insulin will be discussed.

- (b) The production of an alkaline external secretion rich in proteolytic enzymes, lipase and amylase.

#### INSULIN

In 1889 von Mering and Minkowski demonstrated that complete pancreatectomy gave rise to a fatal condition very similar to human diabetes mellitus. Laguesse in 1893 suggested that the islets of Langerhans produced an internal secretion, and in 1902 Ssobolew showed that ligation of the pancreatic duct caused atrophy of the acinar tissue but not of the islets and that diabetes did not result. Following this finding many investigators attempted to produce a potent extract of the pancreas, but failed largely due to the destruction of the hormone by the proteolytic enzymes of the pancreas.

In 1922 Banting and Best obtained a potent extract by extracting pancreas after ligation of the duct, and also demonstrated that an active extract could be obtained by acid alcohol extraction of the gland. The latter technique forms the basis of all methods of preparation of the hormone, which is known as insulin, and has completely revolutionized the treatment of diabetes mellitus. The hormone was crystallized by Abel in 1926, and the crystalline material is now generally used in medicine.

For methods of preparation the student is referred to larger texts dealing with the subject.

## (A) PROPERTIES OF INSULIN

Insulin as prepared by modern methods is a white crystalline protein, containing a very small amount of zinc. The crystalline zinc insulin is rather insoluble in water, but is completely soluble at pH 3, and hence is usually dissolved in N/1000 HCl. The isoelectric point is at pH 5.2–5.4 and the material redissolves at a pH of about 7.2. In acid solution it is relatively stable to oxidizing agents, but is rapidly inactivated by reducing agents such as cysteine. It is also rapidly inactivated by heating at a pH of 8.5 or higher. It is rapidly hydrolysed by pepsin and chymotrypsin, but is less effectively attacked by trypsin.

The crystalline material is one of the purest proteins ever prepared; although in some preparations it is contaminated by small amounts of glucagon, more recent preparations have been free of this substance. The activity is usually of the order of 24 units/mg., but some preparations of 28 units/mg. have been claimed.

It is laevo-rotatory and from recent work it appears that the basic molecular weight is about 6,000, the molecule consisting of two amino acid chains linked by S-S bonds. However these units apparently polymerize readily, and most physical methods give a figure of 12,000 rising in multiples of 6,000 to 48,000 according to the conditions of the experiment. At physiological pH the figure obtained is usually 36,000. It has been demonstrated in blood and urine by methods utilizing extremely sensitive biological assays.

For further details of the structure of insulin the student is referred to the work of Sanger and Craig.

Crystalline insulins from various species were recently compared by Craig using an amino acid assay technique of great sensitivity. They were found to differ very slightly in structure, there being generally a difference in a single amino acid. This difference had no effect on the biological activity of the material. Whether the allergic reactions some individuals suffer on injection of insulin from one species, but not if the hormone is derived from another species, are due to these slight differences or whether they are due to the slight impurities in the preparations cannot be answered at this time.

In 1936 Hagedorn and his associates introduced the substances

protamine insulin and protamine zinc insulin in the treatment of diabetes mellitus. These substances are prepared by the precipitation of insulin with protamine in the presence and absence of zinc. They are both more slowly absorbed than regular insulin, the complex with zinc being slower to absorb than the zinc free complex. It generally has little effect in the first two hours, reaches its maximal effect in about twelve hours, and persists for over twenty-four hours. Owing to the delayed action care has to be taken to prevent hypoglycaemia from occurring during the night. Recently other delayed action insulins, notably globin insulin and N.P.H. insulin, have been introduced. These also have a delayed action but it is not as prolonged as that of protamine zinc insulin and hence the danger of nocturnal hypoglycaemia is lessened. The amount of zinc in all these preparations is too small for any toxic effects to be seen.

## (B) CONTROL OF INSULIN SECRETION

### (1) NERVOUS

The pancreas has a nerve supply from the vagi but it appears that this factor does not play any major role in the control of insulin secretion.

### (2) BY THE BLOOD SUGAR LEVEL

It has been demonstrated by Gellhorn and Feldman in the dog, and by Bornstein and by Gruen in man, that the insulin content of the plasma is roughly proportional to the blood sugar level.

Anderson and her co-workers have shown that an isolated perfused pancreas secreted insulin at a rate which was directly proportional to the glucose level in the perfusing fluid up to a level of about 180 mg./100 ml. at which level peak values were obtained. Hence it would appear that insulin secretion is directly controlled by the level of glucose in the blood, although other factors may well play a part.

### (3) HORMONAL

It has recently been shown by Haist and others that growth hormone is a trophic factor for the beta cells, but the significance of this finding has not yet been established.

### (C) MODE OF ACTION OF INSULIN

The outstanding feature of the action of insulin is its ability to lower the blood sugar level. The extra glucose so utilized is laid down as glycogen, enters fat and protein synthesis or is oxidized. A further action of insulin appears to be to inhibit neoglucogenesis, although this may well be secondary to the acceleration of protein synthesis.

Thus in diabetes mellitus the principal features are:

- (a) Inability to properly utilize glucose.
- (b) Inability to synthesize glycogen, fat and protein.
- (c) Excess neoglucogenesis.
- (d) As glucose is being inefficiently utilized, the energy requirements of the body are met by mobilization of depot fat and its later oxidation. As the oxidation is, in the absence of glucose metabolites, inefficient, this mobilization leads to a lipaemia and accumulation of ketone bodies in the system.

It must be pointed out however that diabetic tissues are capable of oxidizing glucose, although studies of the respiratory quotients suggest that non-carbohydrate substrates form the bulk of the material oxidized by the diabetic organism.

Exactly how insulin accomplishes its action remains in doubt at the present time. Two main theories are held.

Cori and his co-workers have suggested that insulin acts by releasing the enzyme hexokinase from inhibition by anterior pituitary and adrenal cortical secretions, and some experimental evidence in support of this theory has been presented, but generally this evidence is not satisfactory, and many workers have been unable to reproduce the results claimed by the original group. A further unsatisfactory feature of this hypothesis is that if it is taken literally then a hypophysectomized, adrenalectomized animal should be insensitive to insulin, whereas such animals are in fact hypersensitive to the hormone. However at the present time none of these facts make it possible to discard this hypothesis and it must be seriously considered.

More recently Levine and his co-workers have shown that certain non-utilizable sugars which are structural analogues of glucose, (galactose, l-arabinose, d-xylose) are actively trans-

ferred from the extracellular compartment to the intracellular compartment under the influence of insulin, and have suggested that insulin acts by facilitating such a transfer, thus making more substrate available for the enzyme system. Experimental evidence confirming the existence of this finding has been presented by a number of other workers notably Drury and Wick, and by Ross. Bornstein and Park have shown that the phenomenon holds for glucose as well as for the above mentioned analogues. However the actual mechanism of the transfer and its relation to insulin antagonists have so far not been studied, and until these relationships have been worked out, the exact nature of the action of insulin must remain obscure. A further point of very considerable interest is the finding of Stadie *et al.* that insulin binds to the cell, and that the degree of activity is to some extent proportional to the amount bound.

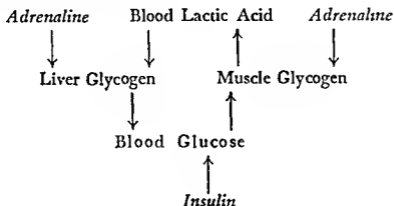
The parts played by under-utilization and overproduction of glucose in producing hyperglycaemia, remain to some degree in doubt, but present data suggest that both phenomena play a considerable role in diabetes, although failure of utilization is the dominant factor.

#### (D) HYPOGLYCAEMIA

In man hypoglycaemia can be induced by injection of insulin or very rarely by natural overproduction such as occurs in secreting tumours of the islet tissue. A form known as functional hypoglycaemia sometimes occurs in normal subjects following the ingestion of carbohydrate, and is at times a very striking feature of the "dumping syndrome" which may follow partial gastrectomy. Hypoglycaemia is also a striking feature in hypopituitarism and in Addison's disease (bilateral destruction of the adrenals, usually tubercular in origin) and may readily be induced in both conditions by fasting the patient. The symptoms are extreme hunger, tremulousness, flushes, perspiration, loss of judgment, delirium and if the state is not relieved, convulsions, coma and death. Prolonged hypoglycaemia even if eventually relieved may lead to considerable cerebral damage. It is deliberately induced in the treatment of schizophrenia.

The hypoglycaemic state can be relieved by the administration of glucose either orally or by intravenous injection or alter-

natively by the subcutaneous injection of adrenaline, the hormone acting by breaking down liver and muscle glycogen, and thus making free glucose available, directly in the case of liver and indirectly in the case of muscle. Here the glycogen is broken down to lactic acid and converted to glycogen in the liver as originally shown by Cori, and amplified by the later work of Dale, Corkill and Marks.



Generally the normal blood glucose level is of the order of 80-100mg./100 ml. in the fasting state, and is maintained at this level during starvation by neoglucogenesis.

Symptoms of hypoglycaemia usually appear at a level between 40 and 60 mg./100 ml. and death may occur if the level falls below 20-25 mg./100 ml.

It has been stated however that in some diabetic patients accustomed to levels of 300-400mg./100 ml. hypoglycaemic symptoms may appear at levels of 100-120 mg./100 ml., particularly if the drop has been very sudden. Conversely patients subject to spontaneous hypoglycaemia as a result of secreting tumours of the islets, may tolerate quite low levels before symptoms occur.

Normally hypoglycaemia tends to correct itself, as a fall in blood sugar stimulates the secretion of the various insulin antagonists, but as previously stated hypoglycaemia must be relieved as soon as possible owing to the risk of inducing severe cerebral damage.

Insulin has a profound but apparently indirect effect on fat metabolism. It has been shown by the use of isotopically labelled glucose that about 50 per cent of the glucose utilized by the

tissues is eventually converted into fat. In the diabetic state, fat metabolism is grossly disturbed. There is virtually a total failure of fat synthesis in the severe cases, and fat is mobilized from the depots, giving rise to a lipaemia and carried to the liver where it is oxidized. However this oxidation is not complete, leading to the appearance of abnormal quantities of acetoacetic acid and Beta-hydroxybutyric acid in the blood, and both acids, and acetone (a breakdown product of the acids) in the urine.

### (E) STANDARDIZATION OF INSULIN

The standard preparation consists of pure insulin crystals prepared on behalf of the World Health Organization and kept for distribution at the National Institute for Medical Research, Mill Hill, London. The unit of insulin is the specific insulin activity of one twenty-second ( $1/22$ ) of a milligramme of this standard preparation. Insulin is assayed biologically and this is a complex procedure. In general, the greater the number of units used clinically the greater the fall in blood sugar, but the fall is not directly proportionate to the amount of insulin administered. The glucose equivalent varies considerably, even in the same patient from time to time, so that no accurately fixed glucose equivalent of insulin is possible.

### TESTS FOR PANCREATIC EFFICIENCY

These tests are designed to determine the efficiency of the secretion of:

- (a) insulin,
- (b) the external secretions of the pancreas. The tests given only represent a sample of the various tests which have been devised.

#### *Tests for the Efficiency of Secretion of Insulin*

##### (1) THE GLUCOSE TOLERANCE TEST

*Principle.*—Blood sugar is estimated following a twelve hour fasting period, and then at half-hourly intervals for two and a half hours, after the ingestion of 1 gram of glucose per kilo. of body weight. In practice for adults 50 grams of glucose are routinely used.

*Procedure.*—50 grams of glucose dissolved in 150 ml. of water are given to the patient after a twelve hour fast. Blood is taken for analysis just before the administration of the glucose and at half-hourly intervals thereafter for two or two and a half hours.

Many methods have been described for the estimation of blood sugar. Of these the most accurate are the enzymatic methods, as reducing substances other than glucose do not react with the specific enzymes used. However, at the present time these techniques are not suitable for routine use. All other methods are dependent on the fact that glucose is a reducing substance. Two of these methods are described.

(i) *The Method of Hagedorn and Jensen*

The principles on which this technique is based are:

1. deproteinization of blood by zinc hydroxide,
2. addition to the protein-free filtrate of a known volume of alkaline potassium ferricyanide, part of which (an amount equivalent to the glucose present) is reduced to potassium ferrocyanide while the glucose is oxidized to gluconic acid and other oxidation products,
3. precipitation of the ferrocyanide by the addition of zinc sulphate and sodium chloride and formation of iodine equivalent to the excess ferricyanide from potassium iodide,
4. titration of the iodine with sodium thiosulphate.

*Reagents.*—NaOH, 0.1 N.

Zinc sulphate, 0.45 per cent.

Alkaline potassium ferricyanide. This contains:

Potassium ferricyanide, 1.65 gm.

Sodium carbonate (fused), 10.6 gm. Water to one litre.

Iodide-sulphate-chloride solution.

Sodium chloride, 250 gm.

Zinc sulphate, 50 gm.

Water to 900 ml.

Potassium iodide, 25 per cent (prepared daily).

One part of KI solution is mixed with nine parts of the  $\text{ZnSO}_4\text{-NaCl}$ .

Starch indicator, 1 per cent in saturated NaCl.

Acetic acid, 3 per cent.

N/200 sodium thiosulphate (diluted from N/10, daily).

*Procedure.*—Measure 1 ml. of 0.1 N sodium hydroxide and 5 ml. of 0.45 per cent zinc sulphate into a small dry test tube. Add 0.1 ml. of blood, rinsing out the pipette with part of the contents of the tube. Place the tube in a boiling water bath for three minutes. Filter through a filter paper (*previously washed three times with boiling water*) into a test tube. Wash the first tube and precipitate twice with 3 ml. of hot water.\*

Add 2 ml.—very carefully measured—of alkaline potassium ferricyanide solution.

For the blank take another test tube and carry out the previous steps, omitting only the blood.

Place both tubes in a boiling water bath for 15 minutes, and then immerse them in cold water for a further three minutes. To each tube add 3 ml. of the iodide-sulphate-chloride solution. Stir, and then add 4 ml. of 3 per cent acetic acid. After stirring, titrate with 0.005N sodium thiosulphate from a micro burette. When the solution is a pale yellow colour, add a few drops of a 1 per cent solution of soluble starch in saturated sodium chloride. The titration is completed by adding sodium thiosulphate until the blue colour developed by the addition of starch becomes colourless.

N.B.—Read the end point against a *white* background.

It has been found experimentally that the equivalence of ferricyanide and glucose can be expressed by the equation

$$G = 0.1735 K + \frac{0.0050}{2.27} \cdot K^2$$

G = glucose in mg. K = ml. of 0.005 N ferricyanide.

However use of this calculation is tedious and two simplifications have been arrived at for general use.

(a) For blood sugar levels in the normal or slightly raised range, the difference between the titration figure and the blank (K) is multiplied by 0.177.

(b) A table (see below) has been prepared covering the range

\* Test may be safely left here for completion later.

from 0.385 mg./100 ml. This table covers most general needs, but if the blood sugar be higher than 385 mg./100 ml., the basic formula should be used to calculate the result. In using the table the glucose equivalent of the blank is subtracted from the glucose equivalent of the unknown, to give the answer.

The principal advantages of this method are that a very small amount of blood (0.10 ml.) is required for each estimation, and that large numbers can be dealt with simultaneously, and as the conditions of heating are not absolutely critical, numerous standards with each estimation can be avoided.

TABLE V  
Hagedorn and Jensen Blood Sugar Table

Hundredths of 1 ml. of 0.005N  $\text{Na}_2\text{S}_2\text{O}_3$ .

ml. of 0.005N $\text{Na}_2\text{S}_2\text{O}_3$	0	1	2	3	4	5	6	7	8	9
0.0	385	382	379	376	373	370	367	364	361	358
0.1	355	352	350	348	345	343	341	338	336	333
0.2	331	329	327	325	323	321	318	316	314	312
0.3	310	308	306	304	302	300	298	296	294	292
0.4	290	288	286	284	282	280	278	276	274	272
0.5	270	268	266	264	262	260	259	257	255	253
0.6	251	249	247	245	243	241	240	238	236	234
0.7	232	230	228	226	224	222	221	219	217	215
0.8	213	211	209	208	206	204	202	200	199	197
0.9	195	193	191	190	188	186	184	182	181	179
1.0	177	175	173	172	170	168	166	164	163	161
1.1	159	157	155	154	152	150	148	146	145	143
1.2	141	139	138	136	134	132	131	129	127	125
1.3	124	122	120	119	117	115	113	111	110	108
1.4	106	104	102	101	99	97	95	93	92	90
1.5	88	86	84	83	81	79	77	75	74	72
1.6	70	68	66	65	63	61	59	57	56	54
1.7	52	50	48	47	45	43	41	39	38	36
1.8	34	32	31	29	27	25	24	22	20	19
1.9	17	15	14	12	10	8	7	5	3	2

Convert both titrations to milligrams of glucose and subtract value of "blank" from patient's sample. The result is expressed as Milligrams Glucose/100 ml. Blood.

The disadvantage is that it does not give a true blood glucose level although it is adequate for most clinical purposes. Owing to the fact that glutathione, uric acid and phosphorylated intermediates of glucose also reduce ferricyanide, and are not pre-

precipitated in the deproteinization used, the value obtained is 20-25 mg./100 ml. above the true glucose level as specifically estimated by either glucose oxidase, or the hexokinase-glucose-6-phosphate dehydrogenase-Coenzyme 2 system. Although this is no handicap in general work, it introduces a serious error in states of severe hypoglycaemia.

Accordingly a number of "true" glucose methods have been devised for routine use. These techniques which are nearly all based on the work of Somogyi, give values only 1-3 mg./100 ml. above the specific enzymic estimations and for all practical purposes the levels obtained may be assumed to represent the true glucose value. In the method described, glutathione and the phosphorylated intermediates are precipitated as barium salts, and uric acid does not reduce the reagent. The great disadvantage is that as boiling conditions are absolutely critical, the reaction must be carried out in standard tubes, immersed to a standard depth in the boiling bath and that two standards have to be run with each batch. Timing must also be very accurate. However with practice the method is easily handled and in its colorimetric modification is probably the most rapid method of all.

#### (ii) *Estimation of Glucose in Blood (Somogyi—Nelson)*

*Principle.*—The haemolyzed blood after treatment with barium hydroxide is deproteinized with zinc sulphate, the volumes of barium and zinc solutions being so adjusted that the final mixture is slightly alkaline, and the mixture is then centrifuged. An aliquot of the supernatant is then boiled with an alkaline copper (cupric) solution which is reduced to an insoluble cuprous salt. The glucose is then estimated either by titration after addition of iodide or colorimetrically by the addition of arsenomolybdate which gives a blue colour. The colorimetric technique of Nelson is described.

*Reagents*—(a) Deproteinizing solutions.

(i) 0.3 N barium hydroxide.

(ii) 5 per cent zinc sulphate ( $\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$ )

The two solutions are titrated and adjusted so that 4.7 ml. of barium hydroxide are just equivalent to 5.0 ml. of zinc sulphate (Phenolphthalein end point). The barium solution is stored protected from atmospheric  $\text{CO}_2$ .

## (b) Alkaline copper solution.

- 4 gm.  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
- 24 gm.  $\text{Na}_2\text{CO}_3$  (anhydrous)
- 16 gm.  $\text{NaHCO}_3$
- 12 gm. Rochelle salt
- 180 gm.  $\text{Na}_2\text{SO}_4$  (anhydrous)
- Water to 1 litre.

The reagent is prepared as follows:

The carbonate and Rochelle salt are dissolved in about 250 ml. of water, and then the dissolved copper sulphate added followed by the bicarbonate. The sodium sulphate is dissolved in about 500 ml. of water, boiled to expel air. After cooling the two solutions are mixed and diluted to 1 litre.

During the first week some auto-reduction occurs and the cuprous oxide is filtered off. After this time the reagent is stable at room temperature.

## (c) Arsenomolybdate reagent.

Dissolve 25 gm. ammonium molybdate in 450 ml. water, add 21 ml. of concentrated sulphuric acid, mix, and then 3 gm. of sodium arsenate ( $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ ) dissolved in 25 ml. of distilled water, mix. Store at  $37^\circ\text{C}$  for 24-48 hours and then at room temperature in a dark bottle.

*Technique of Blood Sugar Estimation*

1. Take 0.1 ml. blood and add to a centrifuge tube containing 2 ml. of distilled water.
2. Add 0.2 ml. of  $\text{Ba}(\text{OH})_2$  solution and shake.
3. Add 0.2 ml. of the zinc solution, and shake vigorously. A white precipitate of barium sulphate, protein and other precipitated material forms.
4. Centrifuge.
5. With cotton wool tipped pipette take 1 ml. of the supernatant liquid and transfer to a standard boiling tube graduated at 10 ml.
6. Add 1 ml. of the alkaline copper reagent. Cap the tube.
7. Place in boiling water bath for 10 minutes.
8. Cool in water for 3 minutes.

9. Add 1 ml. of the arsenomolybdate reagent, and shake until all effervescence ceases. Stand for 5 minutes to allow the blue colour to fully develop. The colour is fast for about 40 minutes and then begins to fade.
10. Dilute to the mark, and read in a photoelectric colorimeter at 540 m $\mu$  or using a green filter.
11. Blanks are prepared by going through the entire procedure omitting the blood, and standards by boiling at the same time 1 ml. of a glucose solution containing a known amount of glucose, usually 100 micrograms.

*Calculation:*

$$\frac{x}{S} \times 0.1 \times 2.5 \times \frac{100}{.1} = \text{mg. Glucose / 100 ml. blood}$$

$$\text{i.e. } \frac{250x}{S} = \text{Glucose in mg. per cent}$$

where x is the reading of the unknown against the blank, and S is the reading of a 100 microgram standard against the blank.

## (2) CLINICAL SIGNIFICANCE OF THE GLUCOSE TOLERANCE TEST

Using Hagedorn and Jensen's method or the Somogyi method it is generally found that the fasting blood glucose level is of the order of 80-100 mg./100 ml., the lower value being obtained in the "true" sugar method, and rising to a level of not above 170 mg. per cent during the first hour and returning to normal within two hours of the commencement of the test. Frequently it is found that the level at two hours is a little below the fasting level and returns to the fasting level at two and a half hours. No glycosuria occurs at any time. The rise in blood sugar is due to the absorption of glucose from the gut into the circulation and the fall to the rapid entry into cell metabolism under the influence of insulin secreted as a result of the rise in blood sugar.

In the diabetic patient the fasting blood sugar is almost invariably raised to above 130 mg. per cent and may be very much higher. After taking 50 gm. of glucose the blood sugar rises rapidly to well above 180 mg. per cent and may rise as high as 400-600 mg. per cent. The curve may not attain its maximum for nearly three hours, and the high levels may be maintained for several hours longer. Under these conditions glycosuria is invariably found.

In some normal individuals a so-called lag curve is observed. Here the blood sugar begins at the normal fasting level but rises to above 180 mg. per cent, causing some glycosuria, but returns to normal within the two hours. Such curves are often found in very obese individuals, and can be restored to normal by placing the patient on a well-balanced diet.

An interesting type of curve is something observed in patients who are suffering from the "dumping syndrome" which occurs at times after partial gastrectomy. Here the blood sugar rises rapidly from a normal fasting level to above 200 mg. per cent and then falls precipitately into hypoglycaemic levels at times giving rise to quite severe symptoms.

In hypopituitarism, the curve may be flat or it may rise from a rather low fasting level to about 120 mg. per cent and then be followed by a rapid fall into hypoglycaemic levels, and symptoms of hypoglycaemia may be quite severe.

In Addison's disease (bilateral destruction of the adrenals) the curve is usually quite flat which has led to the suggestion that adrenal steroids are necessary for the absorption of glucose.

As mentioned in the previous chapter, if a low renal threshold be present glycosuria would be observed in the presence of a normal glucose tolerance test. Typical curves are shown in the accompanying charts (Figs. 16, 17).

The glucose tolerance test is the most valuable biochemical aid in the diagnosis of diabetes mellitus. It is the only method by which renal diabetes can be differentiated, and can be of considerable assistance in association with other investigations in the diagnosis of other endocrine disorders. It must be emphasized that if the fasting blood sugar be above 160 mg. per cent as determined by the Hagedorn method or above 130 mg. per cent by any of the "true" blood sugar methods the patient is diabetic and the glucose tolerance test is only of academic interest. The test is only of use in the diagnosis of diabetes, and is of no assistance in treatment; the best method of determining whether the patient is stable on a given insulin dosage and/or diet is the performance of blood sugars at various times during the day while the patient remains on his or her usual daily routine.

Inquiry into the patient's previous diet may avoid occasional errors in interpretation, as patients who have been taking a very

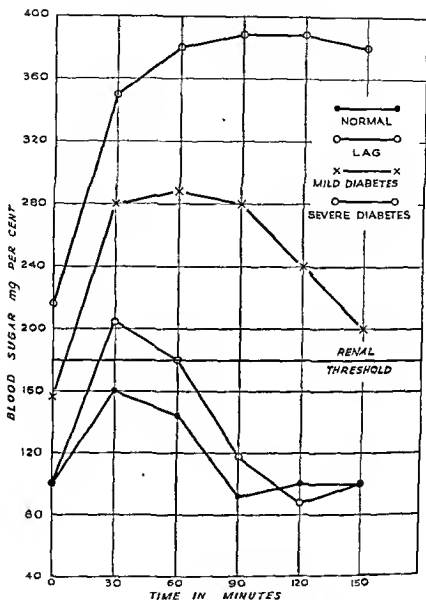


FIG. 16. Glucose Tolerance Curves.

high fat diet for some time frequently present with a diabetic type curve, which disappears when a balanced diet is given for a short period.

### (3) FASTING BLOOD SUGAR IN PREGNANCY

Statements concerning the percentage of sugar in the blood of a fasting patient in pregnancy are conflicting. Rowley, Schiller and Morris independently suggest 80-120 mg. per cent glucose

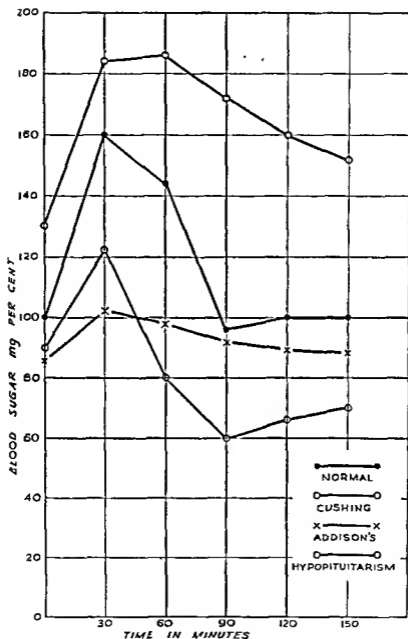


FIG. 17. Glucose Tolerance Curves in Endocrine Disorders (not always obtained, have to be evaluated with other findings).

as normal for the healthy pregnant woman. Nayar found the figure to be below 70 mg. per cent in 65 per cent of his cases. Weiden, working at the Women's Hospital, Melbourne, found the average value to be 85 mg. per cent. In 38 per cent the value

was below the average. In 24 patients tested during and after pregnancy, the blood sugar was higher in the non-pregnant state in one half of them.

#### (4) GLUCOSE TOLERANCE CURVES IN PREGNANCY

##### *Group (1)—No Glycosuria*

In these patients the curve was characterized by:

- (a) Frequency of lowered fasting blood sugar.
- (b) Lowered maximum value.
- (c) Decreased range between fasting and the maximum blood sugars throughout pregnancy.

After the conclusion of pregnancy normal values were found in 70 per cent of this group.

##### *Group (2)—Glycosuria*

Glycosuria occurs frequently in pregnancy, and is of three types: (i) lowered renal threshold, (ii) lowered renal threshold with "superimposed diabetes," (iii) alimentary glycosuria.

- (i) *Lowered renal threshold.* These curves are characterized by:

- (a) Lowered maximum value in 70 per cent of cases.
- (b) Decreased range between fasting and maximum values in 35 per cent.

After pregnancy the same type of curve seems to persist for at least twelve months.

- (ii) *Lowered renal threshold with "superimposed diabetes."*

These curves are characterized by:

- (a) lowered maximum value in the early part of pregnancy only;
- (b) slightly decreased range between fasting and the maximum value in most of these patients, but a marked decrease in some of them.

The patients with "superimposed diabetes" had glucose tolerance curves with delay in return of the blood sugar to normal.

After pregnancy the same type of curve persists for at least twelve months. Persistence of the curves of type 2 (i) and 2 (ii) after a pregnancy seems to indicate that the pregnancy has

produced at least a temporary alteration of kidney permeability for glucose when in the non-pregnant state.

### INSULIN SENSITIVITY AND INSULIN RESISTANCE

The average diabetic patient is reasonably sensitive to insulin, but it is a well-known finding that two patients of the same age, suffering from diabetes of apparently the same severity may require very different amounts of insulin in order to maintain a normal existence. On the average a patient who has had a total pancreatectomy performed, requires about 40 units of insulin per day. However many diabetic patients require amounts far in excess of this figure, thus suggesting the operation of factors other than lack of insulin. When the insulin requirement is over 200 units per day the patient is said to be resistant to insulin. A number of cases has now been described where the insulin requirement reached more than 1,000 units per day. The cause of insulin resistance in diabetes has not as yet been determined. Certain endocrine states other than diabetes show insulin resistance, the outstanding of these are Bilateral Adreno-Cortical Hypertrophy (Cushing's syndrome) and Acromegaly. Conversely the states of hypopituitarism and Addison's disease show insulin hypersensitivity, and insulin tolerance tests are useful in the diagnosis of these conditions.

#### *Insulin Tolerance Test*

Injections of 0.08 units of insulin per kilo. body weight are injected intravenously into the fasting patient, after an initial blood glucose estimation and the blood sugar followed at fifteen-minute intervals for two hours. The types of curves obtained are shown in Fig. 18.

### THE EXTERNAL SECRETION OF THE PANCREAS

Pancreatic juice is alkaline in reaction due to the presence of sodium bicarbonate in approximately deci-normal concentration. In addition to water, salts and a trace of protein, the pancreatic secretion contains the following enzymes.

#### (i) *Trypsinogen and chymo-trypsinogen*

These are proferments or zymogens. Enterokinase of the succus entericus converts trypsinogen into trypsin, but has no

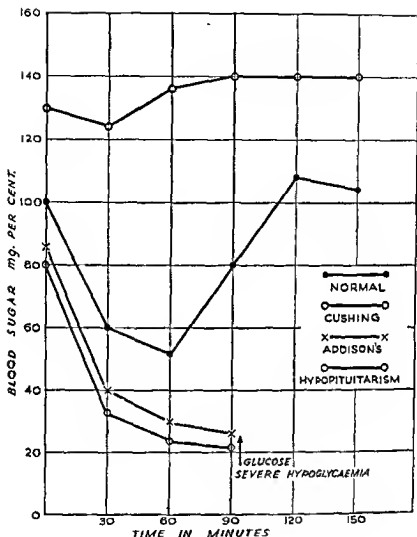


FIG. 18. Insulin Tolerance Curves in Endocrine Disorders (0.08 u. insulin/kilo. body weight fasting).

action on pure chymo-trypsinogen, which is, however, converted into chymo-trypsin by the action of trypsin. Chymo-trypsin clots milk and hydrolyses caseinogen. Both trypsin and chymo-trypsin have been obtained in crystalline form. Trypsin is a powerful proteolytic (proteoclastic) enzyme converting proteins into polypeptides and amino acids. The term "trypsin" is actually applied to a mixture of several proteoclastic enzymes, such as trypsin, carboxypolypeptidase, amino-polypeptidase and protaminease.

(ii) *Lipase or Steapsin*

This is a lipoclastic enzyme converting fats into fatty acids and glycerol.

(iii) *Diastase or amylopsin*

This very powerful amylolastic enzyme transforms starch into various dextrans and then into maltose. It has been shown to have the power of hydrolysing 20,000 times its own weight of starch in 30 minutes at 40°C. Amylopsin will digest raw starch, such as is found in maize, wheat or potatoes. It is not present in the pancreatic juice of infants during the first few months of life, indicating that starchy food is not a normal diet for this period.

(iv) *Maltase*

Converts maltose into glucose.

*Tests for Efficiency of the External Secretion of the Pancreas*

*Introduction.*—Two methods of investigating the activity of the external secretion of the pancreas are in use and are referred to as (i) the direct method, and (ii) the indirect method. In the first method a duodenal tube is allowed to pass into the duodenum and specimens are aspirated by means of a syringe. These are examined for the presence of lipase, amylopsin and trypsin, and from this investigation useful information as to the condition of the pancreatic secretion may be obtained. In the indirect method the faeces are examined for evidence of incomplete digestion of food constituents, especially protein and fat.

(1) EXAMINATION OF FAECES FOR EXCESS OF UNDIGESTED PROTEIN (CREATORRHOEA)

*Procedure.*—A little faeces is rubbed up with water in a mortar to form a paste, and a drop or two of this is transferred to a slide, then stained with carmine, and examined microscopically.

Most European and American authorities state that in normal persons, if muscle fibres are found in the faeces, the muscle striations will have disappeared, owing to the digestive action of trypsin. The faeces of normal Australian people, however, may contain a moderate amount of muscle fibre with striations readily detected microscopically. This may be due to the large

quantity of meat consumed in this country. For this reason it is wise in testing this aspect of pancreatic efficiency in Australian people, to have a standard test diet, such as that of Hawk (p. 340). A charcoal biscuit given before, and at the conclusion of the diet, serves to differentiate the test period. If, on such a diet, a large amount of muscle fibre, with marked striation, is present, then one can infer diminished pancreatic activity.

The more elaborate search for cell nuclei, according to the Schmidt technique, is scarcely necessary and has doubtful diagnostic value.

## (2) EXAMINATION OF FAECES FOR EXCESS OF UNDIGESTED FAT (STEATORRHOEA)

Neutral fat (unsplit) must be hydrolysed by lipase into fatty acid and glycerol before it can be absorbed from the intestinal tract. If the external secretion of the pancreas be diminished, then much neutral fat may appear in the faeces, due to lack of the necessary hydrolysing enzyme lipase. The excess of fat in the faeces due to pancreatic disturbance must be distinguished from that associated with blockage of the common bile duct. In this latter condition hydrolysis of the fat occurs, but the products are not absorbed owing to the absence of bile from the intestine, hence, excess of fatty acid and soap (split fat) appears in the faeces. "Total fat" in faeces includes all substances soluble in ether, together with fatty acids liberated from soap by acid. "Unsplit" fat includes neutral fat, sterols and pigments and represents the difference between the total and the split fat. "Split" fat includes preformed fatty acids and fatty acids liberated from soap by hydrolysis. According to Harrison the following generalization may be made for normal children. "Of the dried faeces not more than one-third (33 per cent) should be fat, and of that (faecal fat) not more than one-third should be unsplit, remembering that in infants even one-half of the dried faeces may be fat." In normal adults, "of the dried faeces not more than one-quarter (25 per cent) should be fat, and of that (faecal fat) not more than one-third should be unsplit". The ratio of unsplit to split fat (free fatty acid, plus soap), is all important as it indicates the efficiency of fat digestion. The total fat gives an estimate of the efficiency of absorption. It may be 50

per cent or more of the faeces in obstructive jaundice. Liquid paraffin must not be ingested for several days before collecting faeces for fat analysis. Estimation of fat in faeces may be conveniently performed by the method of Holt, Courtney and Fales.

### (3) ESTIMATION OF DIASTASE IN URINE

(Dodd's modification of the Wohlgemuth technique)

*Introduction.*—The origin of diastase in the urine is not known with certainty, but it has been considered as arising from the pancreas, and, after passing into the alimentary canal, by way of the pancreatic duct, it is absorbed into the circulation, carried to the kidneys, and finally excreted in the urine. If the kidneys are diseased, the output of diastase in the urine may be less than normal, and this has been used as a test of renal efficiency, but has not been found very reliable. In acute pancreatitis, the diastase in urine is markedly increased (during the height of the attack), presumably due to direct absorption of the enzyme from the pancreas into the circulation, whence it passes to the kidneys and so into the urine.

*Principle.*—The test depends upon the power of diastase to hydrolyse starch into maltose.

*Reagents.*—1. 0.2 per cent starch solution.

2. Phosphate buffer solution (p. 360).

*Procedure.*—To 8 ml. of the buffer add 2 ml. of fresh urine, mix and distribute in a series of short test tubes, as follows:

No. of Test Tube . . .	1	2	3	4	5	6	7	8	9	10	11	12
Buffered Urine (ml) . .	1.5	1.0	0.5	0.45	0.4	0.35	0.30	0.25	0.20	0.15	0.10	0.05
Distilled water (ml.) . .	0.5	1.0	1.5	1.55	1.6	1.65	1.70	1.75	1.80	1.85	1.90	1.95
0.2% Starch Solution (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

The urine is added first, then the water, followed by the starch solution. Finally, 1 ml. of water is added to each tube, taking care to wash the sides of the tubes as the water is de-

livered. The tubes are placed in a water bath or in an incubator for 30 minutes at 37°C, then cooled and sufficient N/50 iodine added to each tube to give a faint blue colour if starch be present.

**Calculation.**—A unit of diastase is that amount which just digests 1 ml. of 0.1 per cent soluble starch at 37°C in 30 minutes to such a degree that no blue colour is obtained on adding iodine.

$$\text{number of diastase units in 1 ml. of urine} \\ = \frac{2}{\text{smallest volume of urine not giving a blue colour}}$$

Thus if tube 10 is the first one to show a blue colour with iodine then the smallest volume of urine just able to digest 1 ml. of 0.2 per cent starch is that in tube 9. This volume is 0.2 ml. of buffered urine, which is a 1 in 5 dilution of the original urine, or 0.04 ml. of original urine.

Hence, number of diastase units per ml. of urine  $\approx \frac{2}{0.04} = 50$ .

**Clinical value of the diastase test.**—The normal range for urinary diastase is from 5 to 20 units. Dodds and other chemical pathologists have found that in acute haemorrhagic pancreatitis, the diastase content of the urine is markedly increased, usually a figure of 200 units or more being obtained. This test serves as a valuable means of distinguishing acute pancreatitis from other acute abdominal conditions having similar clinical signs and symptoms.

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## VI

### ACIDOSIS

#### INTRODUCTION

The hydrogen ion concentration of the blood plasma depends on the relative proportion of H and OH ions in the blood. An excess of hydrogen ions is called acidæmia, whilst an excess of hydroxyl ions is termed alkaliæmia. The two chief substances responsible for these ions are (a) dissolved  $\text{CO}_2$  or  $\text{H}_2\text{CO}_3$ , which ionizes only slightly into H and  $\text{HCO}_3$ , and (b)  $\text{NaHCO}_3$ , which ionizes very readily into Na and  $\text{HCO}_3$ ; the Na interacts with water with the formation of OH ions. This dependence of H ion concentration on the relative proportion of  $\text{H}_2\text{CO}_3$  and  $\text{NaHCO}_3$  in the blood is expressed thus:

$$\text{cH} = k \frac{\text{H}_2\text{CO}_3}{\text{NaHCO}_3}.$$

There are nine possible variations in the components of this ratio. The  $\text{H}_2\text{CO}_3$  may be diminished, normal or increased, whilst the  $\text{NaHCO}_3$  is constant. On the other hand, the  $\text{NaHCO}_3$  may be diminished, normal or increased, while the  $\text{H}_2\text{CO}_3$  remains constant. Or again, both  $\text{H}_2\text{CO}_3$  and  $\text{NaHCO}_3$  may together be increased, diminished, or remain normal. It is the ratio of these two substances that is important and not the actual amount of either. Thus, each may be doubled, trebled or halved, but so long as the ratio is unaltered the resulting pH is unaffected. This is important, since it shows that estimation of either of these components alone does not give a true indication of whether acidæmia or alkaliæmia is present.

Normally the free carbonic acid in the blood amounts to about 3 vols. per cent and the carbonic acid combined with bases to 60 vols. per cent, the ratio being

$$\frac{\text{H}_2\text{CO}_3}{\text{BHCO}_3} = \frac{3}{60} = \frac{1}{20}.$$

So long as this ratio is maintained the reaction of the blood will be normal. Anything which increases the ratio of free carbon dioxide to combined carbon dioxide will result in an acidosis and, conversely, anything which diminishes the ratio of free carbon dioxide to combined  $\text{CO}_2$  will result in an alkalosis.

$$\begin{aligned}\text{Thus: } \frac{\text{H}_2\text{CO}_3}{\text{BHCO}_3} &= \frac{2}{40} = \frac{1}{20} \text{ (normal);} \\ \frac{\text{H}_2\text{CO}_3}{\text{BHCO}_3} &= \frac{2.4}{40} = \frac{1}{16.7} \text{ (acidosis);} \\ \frac{\text{H}_2\text{CO}_3}{\text{BHCO}_3} &= \frac{1.6}{40} = \frac{1}{25} \text{ (alkalosis).}\end{aligned}$$

The term acidosis is used in a somewhat more comprehensive sense than is the word acidaemia. Whilst acidaemia indicates an excess of hydrogen over hydroxyl ions in the blood, acidosis is a term usually used clinically to denote a lowering of the alkali reserve (chiefly  $\text{NaHCO}_3$ ) of the body due to its partial neutralization by abnormal quantities of such acids as  $\beta$ -hydroxybutyric, aceto-acetic and phosphoric acids. For all practical purposes this view of acidosis is true, but there are occasional exceptions which we need not here consider. If the neutralization of acid is satisfactorily performed it may be unaccompanied by increase in H ions in the blood, and the condition is called compensated acidosis. If, however, the mechanism of neutralization is incomplete, then uncompensated acidosis or acidaemia occurs with increase of H ions in the blood. For the condition in which the essential element is the circulation of the so-called acetone bodies, the term ketosis has been applied. Ketosis and acidaemia may co-exist, but this is not necessarily so.

It must be recognized that, although estimation of  $\text{NaHCO}_3$  shortage is largely a measure of the degree of acidosis, yet it measures but one aspect of the compensating power of the body. Thus there may be marked diminution of the plasma  $\text{NaHCO}_3$ , but, owing to a relatively sensitive respiratory centre and normal lungs,  $\text{CO}_2$  may be readily eliminated so that  $\text{H}_2\text{CO}_3$  falls *pari passu* with the fall in  $\text{NaHCO}_3$ . Acidaemia is, therefore, absent,

though the  $\text{NaHCO}_3$  is low. On the other hand, there may be only a small diminution of  $\text{NaHCO}_3$ , but, due to chronic lung disease, compensation by increased loss of  $\text{CO}_2$  may be most ineffective. Acidaemia may therefore be marked. Despite these fallacies, for most clinical purposes the best method of estimating the intensity of acidosis is by determining the alkali reserve of the blood (van Slyke's method) and interpreting the figures so obtained in the light of the clinical examination of the patient.

The clinician usually recognizes the presence of acidosis in a patient by the odour of acetone in the patient's breath, or by such simple qualitative urinary tests for aceto-acetic acid and acetone as those of Gerhardt and Rothera. Furthermore, respiration is usually greatly increased. While these tests are very useful, it must be remembered that in advanced chronic renal disease, acidosis due to retention of phosphoric acid may occur without acetone bodies appearing in the urine, and in such circumstances the estimation of the alkali reserve is of considerable importance.

Table VI indicates the variations in hydrogen ion concentration ( $\text{cH}$ ) and  $\text{NaHCO}_3$  content of the blood plasma under varying conditions.

TABLE VI

Variations of the Hydrogen Ion Concentration under varying conditions

<i>Experimental procedure</i>	<i>Effect on <math>\text{cH}</math></i>	<i>Effect on <math>\text{NaHCO}_3</math></i>	<i>Corresponding clinical condition</i>
Administration of $\text{HCl}$	Increased	Decreased	Acidosis of diabetes and nephritis
Inhalation of $\text{CO}_2$	Increased	Increased	Uncompensated cardiac disease Emphysema
Administration of $\text{NaHCO}_3$	Decreased	Increased	Excessive alkali treatment
Voluntary hyperpnoea	Decreased	Decreased	Anoxaemia of high altitudes Hyperpnoea due to hot baths

The method of expressing reaction in terms of hydrogen ion concentration is indicated in the following description:

A neutral solution is one which contains equal numbers of hydrogen and hydroxyl ions, while an acid solution contains an excess of hydrogen ions, and an alkaline solution contains an excess of hydroxyl ions. Pure water (neutral) is a 1 in 10,000,000 normal acid and a 1 in 10,000,000 normal alkali as well, or to use the logarithmic notation its hydrogen ion concentration is expressed as pH 7. Exponents above 7 indicate alkaline solution and those below 7 indicate acid solution. Normal blood varies from pH 7.3 to pH 7.5. It is therefore slightly alkaline. In very severe acidosis it has been known to fall to 6.98 and, after alkali administration, to rise to 8.0, but these are extreme examples and usually the hydrogen ion concentration of the blood remains remarkably constant.

It should be remembered that the extent to which an acid liberates hydrogen ions determines the ability of that acid to alter the reaction of water to which it is added.

TABLE VII  
Degree of Dissociation of Some Acids and Bases

<i>Acids</i>	<i>% dissociated</i>	<i>Bases</i>	<i>% dissociated</i>
HCl N/10	91.0	NaOH N/10	91.0
Acetic N/10	1.34	KOH N/10	91.0
Carbonic N/10	0.17	AmOH N/10	0.4

A tenth normal aqueous solution of hydrochloric acid and acetic acid each have the same potential acidity, but at any moment 97 per cent of the hydrogen of the hydrochloric acid dissociates and forms hydrogen ions, whereas only 1.3 per cent of the hydrogen of the acetic acid is so dissociated, hence HCl is a much stronger acid—seventy times—than acetic acid. In a similar way, carbonic acid ( $H_2CO_3$ ), which is present in blood, ionizes very feebly and is therefore a weak acid and only slightly influences the reaction of the blood. It is found, however, that even if a strong acid such as hydrochloric acid is introduced into the blood, the resulting increase in hydrogen ion concentration is very slight compared with that which would occur if it were mixed with a similar volume of water instead of blood. This is due to the buffer action of blood or its ability to “mop up” H or OH ions and prevent them from accumulating.

Table VII shows the degree of dissociation of some acids and bases.

## CHEMICAL FACTORS MAINTAINING THE NORMAL REACTION OF THE BLOOD

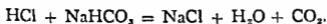
While both lungs and kidneys are concerned with the regulation of the reaction of the blood, the following bio-chemical mechanisms are involved in the maintenance of the normal pH of the blood.

### (1) BICARBONATES OF THE BLOOD

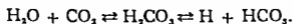
Of the bases in the blood plasma, in combination with weak acid, the major portion exists in the form of sodium bicarbonate, hence this constitutes the alkali reserve of the plasma. The

reaction of the blood is reflected in the  $\frac{H_2CO_3}{NaHCO_3}$  which normally is as 1 to 19 or 20.

Increase in  $H_2CO_3$  or decrease of  $NaHCO_3$  may give rise to acidæmia, diminution of  $H_2CO_3$  or a rise in bicarbonate may induce alkalaemia. If both  $H_2CO_3$  and  $NaHCO_3$  increase or diminish proportionately a normal reaction will be maintained. In acidosis the alkali of the blood is used to neutralize the acids present and the reserve becomes diminished. If a strong acid such as  $HCl$  were introduced into the circulation it would react with the sodium bicarbonate according to the following equation:



The sodium chloride formed in the reaction would be excreted by the kidneys. The water and carbon dioxide may be represented as forming  $H_2CO_3$ , which dissociates feebly, and hence has little influence on the hydrogen ion concentration of the blood:



Furthermore, the carbon dioxide (or slight increase of  $H$  ions) stimulates the respiratory centre and increases pulmonary ventilation, so that it automatically provides for its own removal by increased respiration. Some of this  $H_2CO_3$  may interact with the  $K_2Hb$  in the blood corpuscles, as described in a subsequent

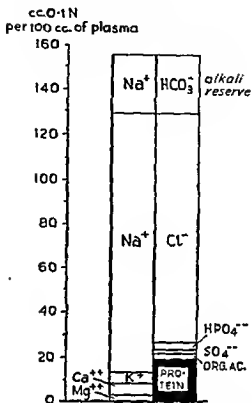


FIG. 19. Diagram illustrating normal acid-base balance. (Modified from Gamble, Ross and Tisdall by Best and Taylor.)

section. The net result of the introduction of HCl into the circulation would be to deplete the blood plasma of some fixed base, but to cause practically no alteration in its hydrogen ion concentration. Lactic acid, which appears in the blood in excess in vigorous exercise is converted into sodium lactate and  $\text{CO}_2$  is liberated. The lactate is subsequently oxidized to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . In a similar manner,  $\beta$ -hydroxybutyric acid would be converted into sodium hydroxybutyrate and removed by the kidneys, while the  $\text{CO}_2$  produced would be eliminated by the lungs. The hyperpnoea accompanying ketosis is thus a protective mechanism. Calculation has shown that an increase of two parts by weight of free carbon dioxide in a million parts of arterial blood, is sufficient to double the breathing during rest.

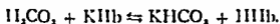
Hartmann has advocated the use of intra-venous injections of isotonic racemic sodium lactate solutions for combating the ketosis associated with diabetic coma. The lactate is slowly

oxidized and the alkali thereby liberated is converted into sodium bicarbonate.

## (2) CHANGES OF BASE ASSOCIATED WITH THE HAEMOGLOBIN AND OXYHAEMOGLOBIN EQUILIBRIUM

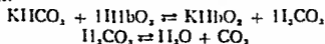
Haemoglobin exists in the blood in an oxygenated and a reduced form. Oxyhaemoglobin is approximately ten times as strong an acid as is reduced haemoglobin. The haemoglobin of the blood corpuscles is combined with potassium and the salt formed may be indicated as KHb.

In the presence of  $\text{CO}_2$  in the capillaries the following reaction occurs:



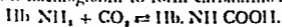
The bicarbonate formed is actually an alkaline substance and the reduced haemoglobin is only weakly acid and dissociates feebly. It has been estimated by van Slyke that 85 to 90 per cent of the base required to carry  $\text{CO}_2$  in the blood is supplied directly or indirectly by haemoglobin. When  $\text{CO}_2$  is diffusing into the blood from the tissues during metabolic activity, it displaces the weakly acidic haemoglobin from its combination with potassium, according to the above equation.

When the blood reaches the lungs, the reduced haemoglobin is oxygenated to the stronger acid  $\text{HbO}_2$ , and  $\text{CO}_2$  is liberated in the blood corpuscle, diffuses into the plasma, and thence to the alveolar air.



This latter reaction is catalyzed by an enzyme, carbonic anhydrase, found in the red blood corpuscles but not in the plasma. It is a protein substance containing zinc, and is readily poisoned by cyanide. If it were not for this catalyzing effect,  $\text{CO}_2$  would not be liberated in sufficient quantities in the short time—one to two seconds—during which the blood is passing through the capillaries of the lungs.

Some 20 per cent of the  $\text{CO}_2$  in the blood corpuscle is combined with haemoglobin to form carbamino haemoglobin.

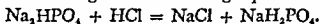


Actually this probably represents 75 per cent of the extra  $\text{CO}_2$  taken up when oxyhaemoglobin is reduced and because of the

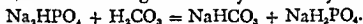
high speed of association and dissociation of this compound is the most mobile part of the  $\text{CO}_2$  carried in the blood. Thus, haemoglobin in its various combinations tends to prevent acidæmia in the blood flowing through the tissues or alkalaemia in the blood in the lungs. Gaseous interchange between red corpuscles and plasma is facilitated by the fact that the corpuscles present a total surface area of more than 3,000 square metres.

### (3) PHOSPHATES OF THE BLOOD

Approximately four-fifths of the phosphate present in the body fluids is in the dibasic form ( $\text{Na}_2\text{HPO}_4$ ). If  $\text{HCl}$  be introduced into the circulation it will combine in part with dibasic phosphate according to the following equation:



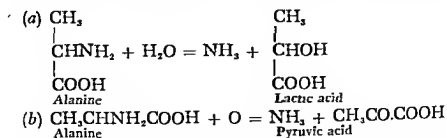
The sodium chloride and acid phosphate so formed are eliminated in the urine.  $\text{H}_2\text{CO}_3$  will also combine with dibasic phosphate, with the formation of sodium bicarbonate and acid phosphate, the latter being excreted by the kidneys.



Thus the kidneys play a very important part in maintaining the neutrality of the blood, usually by excreting acid in the form of acid sodium phosphate.

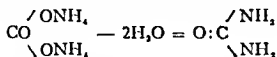
### (4) AMMONIA FORMATION

Amino acids, after absorption from the small intestine, are conveyed to the liver and in part deaminized, possibly by hydrolysis, or more probably by oxidation, with the liberation of ammonia.



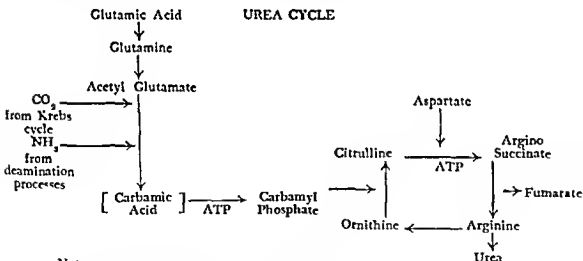
A little of the ammonia so formed combines with strong acid radicals and is excreted in the urine as ammonium salts, while the major portion is converted to urea, which is excreted by the

kidneys. The manner in which ammonia is converted to urea is still a matter of debate. The classical view was that ammonia united with  $\text{CO}_2$  to form ammonium carbonate and this, by the loss of two molecules of water, was converted into urea.



According to Krebs the process is entirely different and depends on the catalyzing action of small amounts of ornithine and on the activity of an enzyme arginase. In the liver ornithine combines with ammonia and carbon-dioxide to form citrulline and this unites with a further molecule of ammonia to form arginine. Arginine is decomposed into urea and ornithine by the action of arginase and the liberated ornithine is now available to repeat the process as illustrated in the following equation:

### UREA CYCLE



#### Note

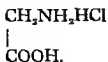
- (1) Multiple function of glutamic acid which acts as:
  - (a) a nitrogen donor;
  - (b) precursor of oxalacetic, and
  - (c) substrate for ATP synthesis via oxidative phosphorylation.
- (2) The  $\text{CO}_2$  fixed in urea is derived from decarboxylation mechanisms—mainly from respiratory processes via Krebs cycle.
- (3) The N atoms fixed in urea are derived from  $\text{NH}_3$ , either *directly* (ornithine  $\rightarrow$  citrulline) or *indirectly* (citrulline  $\rightarrow$  arginine, involving aspartic acid).
- (4) Conversion of ornithine to citrulline occurs in *liver* only; conversion of citrulline to arginine occurs in *both liver and kidney*. Kidney has negligible arginase, so that urea is formed in liver only.

Whilst it is true that in acidosis a large quantity of ammonia may be excreted in the urine in combination with acid radicals, recent research appears to prove that in these circumstances, the quantity of ammonium salts present in the blood shows no increase above normal. Benedict and Nash suggest that ammonia is formed in the kidney (probably from amino acids). This view is supported by their demonstration that the blood of the renal veins contains a larger amount of ammonia than is found elsewhere in the circulation, and also by experiments with isotopic nitrogen. Furthermore, although only a trace of ammonia (0.1 mg per 100 ml.) exists in the circulating blood, a very large amount may appear in the urine without the percentage in the blood being increased.

The ammonia so formed is available for neutralizing acid radicals, and thus spares fixed base in the plasma. This view has been criticized by Briggs who contends that the formation of ammonia is for use as a local protective mechanism to prevent excessive tubular acidity and damage to tubular cells. The experimental evidence, however, does not consistently support this view.

#### (5) PROTEINS OF THE PLASMA

Proteins consist of complex combinations of amino acids which contain the basic radical  $\text{NH}_2$  and the acid group  $\text{COOH}$ , and confer upon the protein molecule amphoteric properties. These amino acids, although in themselves devoid of marked chemical activity, being neither strongly acid nor alkaline, are yet able to act in the presence of strong acids or bases as either base or acid. Thus, with hydrochloric acid, glycine forms glycine hydrochloride:

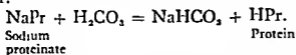


With sodium hydroxide, glycine forms sodium amino acetate.



Some doubt exists as to whether this buffer action of plasma proteins occurs in the living organisms within the narrow limits

of hydrogen ion concentration of the blood compatible with life. As blood is a slightly alkaline solution, the proteins act as weak acids. The plasma proteins are chiefly combined with sodium and this sodium can unite with any acid sufficiently strong to replace the protein molecule. Thus, in the systemic capillaries where  $\text{CO}_2$  is present in abundance, protein is displaced from its combination with sodium, according to the following equation:



The reverse process occurs as the blood passes through the lungs.

#### (6) BASES DERIVED FROM BONE

In extreme cases of acidosis, such as may occur in severe diabetes mellitus, the body may draw on the bases present in bone (Ca and Mg) in an attempt to combat the acidaemia. With modern methods of treatment of diabetes this condition seldom occurs.

### CAUSES OF ACIDOSIS

#### (1) EXCESSIVE FORMATION OF ACETO-ACETIC ACID AND $\beta$ -HYDROXYBUTYRIC ACID

Somogyi, in a review of metabolism of ketone bodies, states: "Concepts regarding the metabolism of ketone bodies have undergone radical changes during the past decade. 'Fats burn only in the fire of carbohydrate' proved to be a catch phrase, invalidated by much conclusive evidence showing that the 'ketolytic' effect of carbohydrates is non-existent. Ample proof has accumulated to show: (a) that  $\beta$ -hydroxybutyric and acetoacetic acids are normal metabolic products; (b) that the liver utilizes very little, if any, of these ketone bodies, while muscle tissues are capable of utilizing considerable amounts of them as a source of energy; (c) that carbohydrate is the preferential source of energy of the liver, in lack of which the liver is compelled to consume increased amounts of ketone-yielding metabolites (fats, amino acids). When, as a consequence, the liver produces ketone bodies at a rate that exceeds the rate of peripheral utilization, hyperketonemia and ketonuria ensue. Deglycogenation of the liver consistently causes ketosis. The action of carbohydrates is only anti-ketogenic in that they

furnish to the liver its preferential fuel and obviate the consumption of large amounts of ketogenic metabolites."

Ketonuria will not occur if the fat metabolized does not exceed twice the amount of carbohydrate plus half the amount of protein ( $F=2C+\frac{1}{2}P$ ). In diabetes aceto-acetic acid (diacetic acid) and  $\beta$ -hydroxybutyric acid are formed in excess, and these, together with acetone which is produced by decomposition of aceto-acetic acid, are called the "acetone bodies." In these circumstances, the acetone bodies appear in the urine and are associated with an excess of phosphoric acid and ammonia. Vomiting in children readily causes acetone bodies to appear in the urine, as also does starvation. The blood may show a diminution of plasma bicarbonate and owing to increased pulmonary ventilation (hyperpnoea) the  $\text{CO}_2$  in the alveolar air is reduced. Even in a condition of most severe acidosis there is but slight alteration in the hydrogen ion concentration of the blood, which is normally pH 7.3 to 7.5. Very small deviations from the normal may cause death, hence there is in the blood provision against any marked change in reaction. These provisions have been discussed in detail, but briefly, they are achieved by (a) the physico-chemical reactions of the blood, (b) the respiratory mechanism, (c) the excretory powers of the kidneys.

## (2) RETENTION OF PHOSPHORIC ACID DUE TO RENAL INEFFICIENCY

In those cases exhibiting renal inefficiency associated with phosphoric acid retention, there is usually no ketosis and a relatively low ammonia content of the urine, but otherwise the biochemical findings of the alveolar air are similar to those in group 1. The low ammonia output may be due to damage of the renal tissues as, according to Benedict, the kidneys are the site of formation of ammonia. It should be remembered that the administration of  $\text{NaH}_2\text{PO}_4$  to render a urine acid is contra-indicated if there is severe renal damage and difficulty in excreting phosphoric acid.

## (3) FAILURE TO EXCRETE CARBON DIOXIDE

Retention of carbon dioxide may occur in uncompensated cardiac disease or in pulmonary conditions in which the ventilation is inefficient, such as is found in pathological states of the

pulmonary alveoli. In some such cases a retention of alkali may occur in an attempt to combat the acidosis.

#### (4) ADMINISTRATION OF ACIDIC SUBSTANCES

Ammonium chloride is frequently administered in therapeutic doses as an adjuvant to the mercurial diuretics. The ammonia portion combines with  $\text{H}_2\text{CO}_3$  and is converted into urea in the liver, whilst the chloride ions react with the bases in the blood, inducing at first a compensated acidosis, but by excessive dosage an actual acidæmia may occur. Calcium chloride in excess may act in a similar manner. It is interesting to note that it seems almost impossible to induce acidæmia by the administration of  $\text{HCl}$  in cases of achlorhydria, such as occurs in pernicious anaemia.

TABLE VIII  
Summary of Causes of Acidosis

<i>Causes</i>	<i>Type</i>	<i>Effect on <math>\frac{\text{H}_2\text{CO}_3}{\text{BHCO}_3}</math> ratio</i>
Severe muscular exercise	Non-gaseous	Reduction of $\text{BHCO}_3$ due to neutralization by lactic acid
Ketogenic diet (High fat diet)	Non-gaseous	Reduction of $\text{BHCO}_3$ due to neutralization by aceto-acetic and $\beta$ hydroxybutyric acid
Diabetic acidosis	Non-gaseous	Reduction of $\text{BHCO}_3$ due to neutralization by aceto-acetic acid and $\beta$ hydroxybutyric acid
Azotaemic (interstitial) nephritis	Non-gaseous	Reduction of $\text{BHCO}_3$ due to retention of acid radicals such as phosphoric acid
Diarrhoea	Non-gaseous	Reduction of $\text{BHCO}_3$ due to excessive excretion of base
Circulatory failure	Gaseous	$\text{H}_2\text{CO}_3$ of blood is increased owing to sluggish transport of $\text{CO}_2$
Pulmonary impairment	Gaseous	$\text{H}_2\text{CO}_3$ of blood accumulates due to inefficient diffusion of $\text{CO}_2$ into lung alveoli
Depression of respiratory centre	Gaseous	$\text{H}_2\text{CO}_3$ accumulates in blood due to inadequate respiration

is negative, provided a freshly voided specimen has been examined.

### (3) ROTHERA'S NITROPRUSSIDE TEST USING A POWDERED REAGENT

Weigh out 20 g. ammonium sulphate, 20 g. anhydrous sodium carbonate and 1 g. of sodium nitroprusside (finely ground). Mix thoroughly but do not grind together.

The mixture should be kept dry and remains effective for many months. When performing the test place a small quantity—about 5 mm. in diameter—on a white filter paper and add one drop of urine. Aceto-acetic acid and acetone will give an immediate violet colour.

### (4) PLASMA KETONE ESTIMATION

*Reagents.*—1. 20 g. ammonium sulphate.

2. 1 g. sodium nitroprusside.

3. 20 g. anhydrous sodium carbonate.

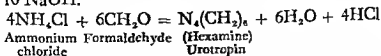
Each is finely ground before mixing and the powder should be kept dry.

*Procedure.*—A small amount of powder is placed on a white filter paper and a drop of serum or plasma added. In the presence of 10 mg. or more ketones per 100 ml. a purple colour develops almost immediately. By diluting the plasma with distilled water it is possible to estimate the plasma ketone concentration to  $\pm 10$  mg. per 100 ml.

### Quantitative Tests

#### (1) ESTIMATION OF THE AMMONIA CONTENT OF THE URINE

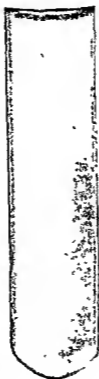
*Principle.*—The estimation of ammonia depends upon the fact that, when a neutral solution of an ammonium salt is treated with formaldehyde, combination occurs with the formation of hexamethylene tetramine (urotropin) and the liberation of a corresponding amount of hydrochloric acid, which is titrated with N/10 NaOH.



Amino acids also react with formaldehyde to give methylene derivatives and the solution becomes acid. The result of the



(a)



(b)



(c)

PLATE V

- (a) Gerhardt's test for aceto-acetic acid (see p. 124)  
(b) Benzidine test for blood (see p. 260)  
(c) Rothera's test for aceto-acetic acid and acetone (see p. 125)



estimation, therefore, gives the sum of the ammonia and the amino acids of the urine, but for clinical purposes this latter factor can be neglected.

*Reagents.*—1. Sodium hydroxide N/10.

2. Phenolphthalein.

3. Neutral potassium oxalate.

*Procedure.*—To 10 ml. of urine add 20 ml. distilled water, 4 or 5 drops of phenolphthalein solution, and 1 gram of neutral potassium oxalate, which is just sufficient to give a faint white precipitate, which helps the reading. Run in NaOH until a faint pink colour is permanent; now quickly add 3 ml. formaldehyde solution (formaldehyde 50 ml. previously neutralized and water 50 ml.) and again run in NaOH until the same pink colour as before is developed. Note the amount of NaOH which has been used in this second titration.

*Calculation.*—Suppose that 3 ml. of N/10 NaOH were used in the second titration; since 1 ml. N/10 NaOH equals 0.0017 grams ammonia, then 3 ml. (the amount assumed to be used in the second titration) NaOH represents  $3 \times 0.0017 = 0.0051$  grams ammonia in 10 ml. urine = 0.051 grams in 100 ml. of urine.

*Clinical value of the test.*—It must be clearly understood that the ammonia estimated is not free in the urine but is present as ammonium salts, the urine as a rule being acid. The normal amount of ammonia excreted per day is 0.7 grams to 1.0 grams. In severe diabetes, the ammonia output may be several grams per day, and this is some guide to the intensity of the acidosis, and as an indication of impending coma, but it has been pointed out by Harrison that "the quantity of ammonia excreted is not necessarily proportional to the risk of acidosis because it is only one of the available mechanisms and may be called into play in varying degree in different patients having a similar grade of acidosis."

Urines must be preserved by the addition of acid (see p. 152). Urines showing bacilluria when passed or which have been allowed to stand without preservative until there is bacterial decomposition of urea with liberation of ammonia are obviously quite unsuitable for this determination.

## (2) ESTIMATION OF THE ALKALI RESERVE (VAN SLYKE)

*Principle.*—Plasma from oxalated blood is shaken in a separating funnel with an air mixture, the carbon dioxide tension of which approximates that of normal arterial blood. A measured quantity of the plasma is now acidified and the carbon dioxide liberated by the formation of a partial vacuum. This carbon dioxide is measured under atmospheric pressure and the volume per 100 ml. of plasma calculated. The greater the quantity of  $\text{CO}_2$  liberated, the greater is the alkali reserve in the form of sodium bicarbonate.

In place of the apparatus described in this section, the manometric apparatus (van Slyke) may be used.

*Reagents.*—1. Phenolphthalein in alcohol (1 per cent).

2. Sulphuric acid (5 per cent).

3. Capryl alcohol.

4. Ammonia 1 per cent.

*Procedure.*—About 10 ml. of blood is collected from a vein by means of a glass syringe, and the blood is delivered into a centrifuge tube containing some powdered oxalate. It is then centrifuged. If a ligature is used to cause venous stasis in the vein, it should be loosened for a few moments before the blood is withdrawn, so as to avoid excess of carbon dioxide from this source.

The plasma is now pipetted off and saturated with  $\text{CO}_2$  at alveolar air tension, in the following manner:

About 3 ml. of plasma are introduced into a dry 300 ml. separating funnel, which is connected by rubber tubing to a bottle full of glass beads. Air is now expired from the analyst's lungs through the bottle and funnel, and the stopper inserted. The moisture condenses on the glass beads and the funnel is filled with alveolar air. The tap of the funnel is closed and the funnel rotated for two minutes, so as to spread the plasma as a thin layer and saturate it with  $\text{CO}_2$  at alveolar tension. The funnel is now placed upright, so that the plasma may drain from the walls and collect in the narrow end.

The apparatus for  $\text{CO}_2$  estimation (see Fig. 20) consists of:

1. 50 ml. pipette (A) with an upper calibrated tube (H).

2. Cup (G) for solutions to be admitted through stopcock (B).

3. Capillary tube (C) for removal of solution from the apparatus.
4. Two-way stop-cock (D).
5. Chamber (E) for drawing off solution after the  $\text{CO}_2$  has been extracted.
6. Chamber (F) for readmitting mercury into (A).
7. Rubber tubing and levelling bulb filled with mercury.

The apparatus, as depicted in the diagram, is tested by filling the system completely with mercury, closing the tap and lowering the reservoir to develop a Torricellian vacuum. It is then raised again. If the taps are airtight a click is heard as the mercury reaches the top of the system. For a  $\text{CO}_2$  estimation the whole apparatus is filled with mercury, including the capillary above the stop-cock B, and the reservoir is placed in a ring support at the level of the stop-cock D. The cup G is washed free

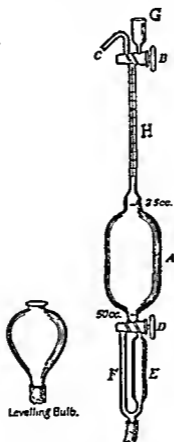


FIG. 20. Van Slyke's apparatus for estimation of plasma  $\text{CO}_2$ .

from any trace of acid by carbonate-free ammonia water, and this is all removed by means of a test and capillary pipette, with the exception of a few drops. One ml. of plasma from the separating funnel is now accurately pipetted into G, care being taken to keep the point of the pipette under the ammonia. The stop-cock B is turned so as to admit the plasma into the calibrated portion, leaving only enough to prevent entry of air through the capillary of the stop-cock. The cup is now washed with two small quantities of distilled water, each being run carefully into the calibrated tube, as was done with the plasma. A drop of capryl alcohol and 0.5 ml. of 5 per cent  $H_2SO_4$  are also run into the pipette. The total volume of fluid in the apparatus should be arranged to be 2.5 ml. The mercury reservoir is lowered about two feet below the cock D, and the mercury allowed to fall to the 50 ml. mark, and D is closed. The pipette is removed from its stand and inverted about 15 or 20 times to ensure liberation of all the  $CO_2$ . It is replaced in the stand and by turning D the fluid is run into E without allowing the  $CO_2$  to follow. The levelling bulb is now raised and mercury passes via tube F and A, and the mercury in the levelling tube and that in the capillary tube are brought to the same level, and the volume of gas (above the trace of water which is always present) is read. The barometric pressure is read and reduced to standard pressure by multiplying by the factor indicated (Table IX) and from Table X can be calculated the  $CO_2$  bound by 100 ml. of plasma.

TABLE IX

Conversion of Barometric Pressure to Standard Pressure

Barometer	Barometer	Barometer	Barometer
	760		760
732	0.961	756	0.995
734	0.966	758	0.997
736	0.968	760	1.000
738	0.971	762	1.003
740	0.974	764	1.006
742	0.976	766	1.008
744	0.979	768	1.011
746	0.981	770	1.013
748	0.984	772	1.016
750	0.987	774	1.018
752	0.989	776	1.021
754	0.992	778	1.024

*Clinical value of estimation of alkali reserve.*—The normal  $\text{CO}_2$  combining power of blood plasma varies from 52 to 79 ml. per 100 ml. of plasma. In mild acidosis, figures from 40 to 45 may be found. Severe acidosis is associated with readings of 30 to 40 and in fatal cases the combining power may be as low as 20, or even less. Such estimations are of considerable value both in prognosis and treatment.

### (3) ESTIMATION OF THE CARBON DIOXIDE CONTENT OF ALVEOLAR AIR

Acidaemia causes compensatory hyperpnoea with resulting fall of the alveolar  $\text{CO}_2$  and therefore of the  $\text{CO}_2$  tension of the blood.

For details of this method see practical physiology books.

### ALKALOSIS

A brief reference only to this subject will be made. An increase of the alkali reserve of the blood is known as alkalosis. Such a condition is not so commonly observed in clinical medicine as is acidosis. Alkalosis may be produced by any condition which diminishes the ratio  $\frac{\text{H}_2\text{CO}_3}{\text{NaHCO}_3}$ .

As discussed at the beginning of this chapter, there are nine possible variations in the components of the ratio, but for clinical purposes the chief causes of alkalosis are: (a) decrease of the carbonic acid of the blood, or (b) increase in the bicarbonate of the blood.

In the first group, hyperpnoea is responsible for diminution of the  $\text{CO}_2$  of the blood, the excessive pulmonary ventilation causing undue loss of  $\text{CO}_2$  from the lungs. This phenomenon is observed at high altitudes, or experimentally under conditions of diminished oxygen pressure. With prolonged immersion in a hot bath a similar hyperpnoea and tendency to alkalosis may develop. In such conditions the output of ammonia in the urine is diminished, as is also the acid phosphate, and there is diminished  $\text{CO}_2$  in the alveolar air.

In the second group, increase of bicarbonate of the blood may be produced by ingestion of bicarbonate or by an excessive loss of  $\text{HCl}$  as the result of vomiting. In such circumstances there is diminished ammonia and phosphate in the urine, excess of

## VII

# WATER AND INORGANIC ELECTROLYTES

## INTRODUCTION

The body normally contains about 60 to 70 per cent by weight of water, or 42 to 49 litres in a person of 70 kg. body weight, the proportion being higher in the young than in the old and in the lean than in the fat. The water moves freely by diffusion in and between the tissues of the body and is the transport medium in blood vessels, bodily channels and interstitial tissues. Approximately two-thirds of the fluid is inside the cells and the remainder, the extracellular fluid (E.C.F.) which bathes the cells, is made up of the interstitial fluid and the plasma.

The amount present in the body can be estimated after introduction of a known amount of heavy water by measuring the dilution of this material. Clinical tests of dehydration and hyperhydration by deforming skin and noting whether it stays wrinkled or shows a slowly recovering depression, while useful, have the disadvantages that they show the state of these tissues only and give unequivocal results only when there is a gross change in these tissues. Even local changes in the subcutaneous tissues do not always reflect a general change which can be inferred only if other tests support the subcutaneous findings. The most important and reliable of such investigations is the variation of weight and the water balance course of the patient.

The water content of a normal person remains remarkably constant over long periods of time and despite great physiological stresses. It is regulated by increase or decrease of intake and output. Water arrives in the body proper by absorption of water ingested either as liquid or in food or by the oxidation of food substances. For temperate climates the figures usually given for these three items are 1,500 ml., 750 ml. and 350 ml. per diem, but the first item varies greatly, sometimes as a result of social customs, (e.g. beer or tea drinking), but also as a result of in-

creased losses. When water is lost from the normally conscious person thirst causes remarkably accurate replacement by drinking.

The channels of loss of water are: the respiratory tract, by saturating the respired air with water vapour; the skin by insensible or actual perspiration; the urine and the faeces. The amount lost from the lungs is usually given as 400 ml. per diem but will be greatly increased by increased pulmonary ventilation, especially of dry air, whether hot or cold. Insensible perspiration is usually estimated at 600 ml. but will be lower in humid conditions and increased in hot, dry conditions. Actual sweating, normally absent, may exceed ten litres per diem when work is done in hot humid conditions. It may be a source of considerable loss in febrile and anaesthetic conditions.

The amount of water excreted by the kidneys, may vary greatly. In order to secrete the non-volatile products of metabolism at maximum urinary concentration a minimum of 500 ml. per diem is needed. Usually more urine than this is formed; about 1,500 ml. is the normal amount for adults, but it may be greatly increased by abnormal intake of fluids or decreased when intake does not compensate for losses through other outlets. The amount and degree of dilution is usually an index of variation of the saline osmotic pressure of the blood plasma and tissue fluid. Verney has demonstrated the mechanism as one of osmoreceptors in the hypothalamus influencing the secretion of antidiuretic hormone via the supraoptico-hypophyseal pathway: destruction of this mechanism gives rise to diabetes insipidus with excretion of up to 40 litres of water in the urine each day. The volume per diem of urine may also be increased by an increase of non-volatile solids to be excreted, e.g. large quantities of sugar and urea in diabetes mellitus. Disorders of the kidney also affect the volume of urine. The amount of water lost in the faeces is usually recorded as 100 ml. per diem but may be less in constipation or greatly increased in diarrhoea. Water may be lost from the body by vomiting, intestinal or glandular fistulae, or from excoriated areas of the surface, e.g. burns. In all these cases where liquid is lost, it is sufficiently accurate to record the volume or weight of the lost material as water.

The approximate water intake and output is summarized in

Table XI. The figures shown are the average for an adult in a temperate climate.

TABLE XI  
Daily Exchange of Water

<i>Intake</i>			<i>Output</i>
As liquid	1,500 ml.	Urine	1,500 ml.
In food	750 ml.	Skin	600 ml.
From oxidation of		Lungs	400 ml.
food	350 ml.	Faeces	100 ml.
	<hr/>		<hr/>
	2,600 ml.		2,600 ml.

The principal electrolytes in the body fluids, tissues and secretions are shown in the block diagrams of Fig. 21. They are expressed as milli-equivalents (m-eq.) per litre of water, which unit is derived from the calculation  $\frac{\text{milligrams per litre} \times \text{valence}}{\text{ionic weight}}$

The advantage of the m-eq. notation is that it expresses directly the chemical equivalence of the material and in a solution the sum of the cationic equivalents is equal to the sum of the anionic equivalents. By leaving out the factor of valence the concentration may be read as milliosmols and the sum of anionic and cationic values gives a direct indication of ionic osmotic pressure.

## WATER AND ELECTROLYTE DISTURBANCES

The subject of water and electrolyte disturbances of the body has been intensively studied in recent years and the amount of information is so great that it compels an abbreviated discussion. The following information should be regarded as an attempt to indicate major features of the problem.

### (1) WATER

(a) *Depletion* of body water may be relative or absolute. Relative depletion is defined as reduction of water in relationship to solids but the total amount may be in excess, normal or less than the normal content of the body. Relative depletion with increased total water may occur when 0.9 per cent NaCl is given intravenously in excessive quantities and the kidney is unable to excrete the NaCl residual from insensible perspiration and respiratory evaporation. Relative water depletion with reduced

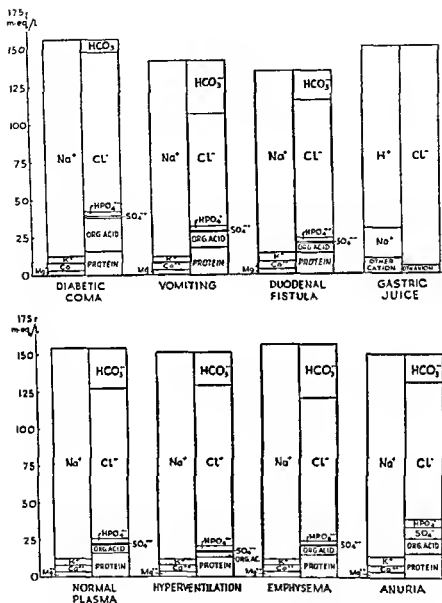


FIG 21. These block diagrams represent the plasma concentrations in actual clinical cases of the several conditions.

body water may occur as result of excessive sweating. In each of these cases Na concentration in plasma will be raised but in the former case body weight will be increased and in the latter it will be decreased. *Absolute* depletion is defined as a diminution of water in relationship to the protoplasmic mass of the body. As seen in the previously quoted example of excessive loss

of hypotonic sweat, the Na or Cl concentration in the blood plasma may be raised while total body water is decreased, as is the total Na and Cl. In diabetes insipidus deprivation of water may lead to absolute depletion without a change in total Na or Cl. In such cases the haematocrit or plasma proteins, or both, will usually show an increase above normal. In planning therapy for water depletion the complexity of these relationships must be taken into account: it is obvious that estimation of one of the variables may lead to over-all error and this is the usual result of applying indiscriminating use of rules which are based on inadequate reasoning.

(b) *Water excess* in the body may similarly be relative or absolute. If the body is losing Na in excess of Cl, as in duodenal fistulae, the kidney excretes Cl in excess of Na and the plasma concentration of both Na and Cl falls in circumstances when the body is actually depleted of water but where the total electrolyte depletion is relatively greater. A similar extent of fall of electrolyte proportion in the plasma may, however, occur when quantities of hypotonic fluid beyond the capacity of the kidney to excrete are introduced into the body, e.g. in acute nephritis or anuria. The previous warning about clinical rules applies in these circumstances.

## (2) SODIUM

Depletion of the body of sodium may occur due to excessive loss and sometimes by inadequate replacement. Excessive loss may occur through diarrhoea, sweating, the kidneys in adrenocortical insufficiency, or pancreatic or salivary fistulae. In civilized conditions inadequate replacement rarely occurs in normal people. The sodium must be lost in association with an anion and the nature of this anion will govern whether there is an associated stress on blood pH. If the sodium is lost in association with bicarbonate there may be acidosis and replacement therapy must aim to correct this deviation as well as the sodium loss.

Sodium excess results from presenting to the body more sodium than the kidney can excrete. The principal factors which affect the kidney are:

- circulatory, e.g., heart failure, shock;
- endocrinal, e.g., hypercorticism;
- or local, e.g., nephritis.

Intravenous "therapy" may be so mishandled by using excessive quantities, especially of high chloride solutions, that a perfectly normal kidney is unable to keep up with the load.

### (3) POTASSIUM

Depletion of potassium usually arises from abnormalities of metabolism, as in diabetic acidosis. When these errors are corrected the plasma potassium may fall to very low levels associated with muscular paralysis and cardiac abnormality: there is some doubt whether the E.C.G. changes which have been described as characteristic of hypokalaemia may not be to some extent due to other associated changes, e.g.  $\text{CO}_2$  level in the blood.

Excess potassium in the extra cellular fluids arises from abnormal behaviour of the kidney and can only occur in cases where there is some degree of impairment of renal function. In anuria the concentration of  $\text{K}^+$  is due to the continuous breakdown of protoplasm and failure of excretion. When potassium citrate is given to alkalinize the non-existent urine the level may rise precipitately to cause sudden cardiac arrest. In adrenal deficiency, as in Addison's disease, the blood potassium rises with the fall of sodium.

### (4) CALCIUM

See chapter on Endocrine Glands, page 245.

### (5) MAGNESIUM

Depletion of magnesium has not been remarked in any particular human syndrome but in puerperal bovines gives rise to a form of general collapse called "grass staggers". Raised blood magnesium occurs in anuria and is due to the liberation of intracellular magnesium and failure of excretion. It may be raised after  $\text{MgSO}_4$  enemata, especially when used in massive quantities in one of the regimens for toxæmia of pregnancy. Drowning in sea water or the ingestion of sea water also may give a high blood magnesium. It is doubtful whether in any of these circumstances the increased magnesium is responsible for any of the symptoms of the condition but levels approaching those giving magnesium anaesthesia in experimental animals have been recorded.

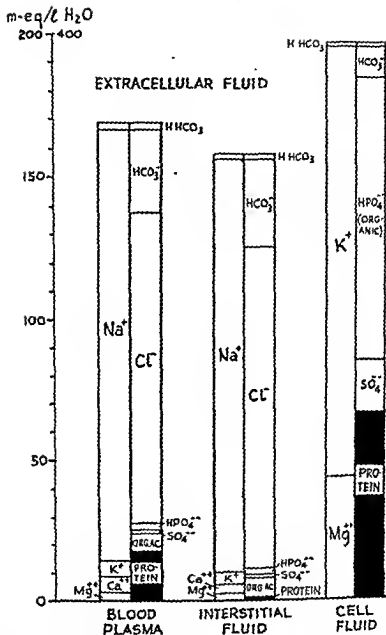


FIG. 22. Electrolyte composition of extracellular fluid and of cell fluid. (From Gamble, *Chemical Anatomy, Physiology and Pathology of Extracellular Fluid*. Harvard University Press.)

#### (6) CHLORIDE

Depletion of the body of chloride usually results from loss of chloride containing secretions or exudates, e.g. gastric juice, sweat, burn fluid. Sweat and burn fluid contain Na and Cl in approximately the proportions present in the blood and so sweating does not disturb the pH of the body, but in gastric

juice  $\text{Cl}^-$  is lost in association with  $\text{H}^+$  and consequently there may be a distortion of the pH pattern with alkalosis. The chloride is also lowered with retention of  $\text{CO}_2$  in emphysema: here the distortion of the pH pattern is acidotic. In anuria the chloride concentration is altered in association with change in other anions in the plasma, but unless there has been sweating or vomiting there is no absolute depletion. When alkaline secretions, e.g. pancreatic or colonic, are lost from the body, they carry away sodium in excess of chloride: in these circumstances the kidney excretes chloride in proportion to the sodium loss, thereby reducing the level below the normal renal "threshold."

Reduction of plasma chloride may also follow use of low chloride intravenous or intra-colonic fluids in excessive quantities.

Clinically, peripheral vascular failure is commonly attributed to reduced plasma chloride but in most instances the chloride effect is a contributory, not the primary cause of this occurrence.

Excess of chloride in the body is usually associated with a raised blood chloride. It may occur in hyperventilation in association with alkalosis. Most commonly it occurs due to intravenous infusion of "normal" saline or Ringer's solution, especially in post-operative or other conditions where the kidney has defective chloride handling capacity. There is no specific clinical sign associated with raised plasma chloride.

#### (7) SULPHATE

Increased amounts and concentrations of sulphate occur in anuria due to the retention of sulphate arising from endogenous metabolism. This may be aggravated by the use of intravenous sulphates given under the illusion that they are diuretic substances.

### GENERAL EFFECTS OF EXCESS WATER

In most circumstances a positive water balance is reflected in increased blood and interstitial fluid volume. The relative distribution of the excess between these two spaces is largely determined by the concentration of plasma proteins and the concentration of these is a very important consideration in planning

liquid replacement. The distribution of excess water between the intracellular and extracellular compartments is principally governed by the relative electrolytic osmotic pressure of the two spaces: therapy which leads to high values in the extracellular space causes shift from the intracellular space and increases the extracellular plethora. If the intravascular material is greatly increased the increased fluid is accommodated in the first instance mainly in the veins. As a result the venous pressure rises but before the veins of the neck of a seated person become obviously filled there may be the onset of oedema in dependent parts. This arises partly as a result of decreased osmotic pressure of blood proteins (which also precipitates pulmonary wetness and even asphyxia) and partly due to increased capillary pressure in the dependent parts. The thoracic cavity is normally below atmospheric pressure and so the veins at the base of the neck and at the diaphragm all drain into this subatmospheric sink: extrathoracic venous pressure is therefore usually atmospheric from the crown of the head to the xiphisternum. As the plethora develops the venous pressure must rise in the chest from the xiphisternum to the manubrium before the classical signs of increased venous pressure arise. This amounts to a rise of approximately 20 cm. of blood or 15 mm. of Hg and there is a consequent rise by this amount in the capillary pressure of the subdiaphragmatic parts. Such a rise, especially if accompanied by reduced concentration of blood proteins will give rise to gross oedema.

## (A) DETECTION OF ABNORMALITY OF CONCENTRATION AND BALANCE

### (1) DIRECT METHODS

#### (i) Concentration

- (1) Water relative to plasma proteins (see pp. 310, 311).
- (2) Plasma relative to red blood corpuscle—haematocrit.
- (3)  $\text{Na}^+$ —Flame Photometer (see below) } See also chapter XV,
- (4)  $\text{K}^+$ —Flame Photometer (see below) } Endocrine Glands
- (5)  $\text{Cl}^-$ —Titration (q.v.) (p. 303).
- (6)  $\text{HCO}_3^-$ —Van Slyke (p. 128).
- (7) Sulphate (Titration).

(8)  $Mg^{++}$  (Colorimetric).

(9) pH. Glass electrode.

(For the last three estimations larger text books should be consulted.)

(ii) *Spatial Distribution of Water*

(1) Total body water. Dilution of injected heavy water.

(2) Extracellular volume. Cl isotope dilution. Na thiosulphate or inulin method.

(iii) *Balance*

Direct measurement of input and output in association with serial body weights.

(2) INDIRECT METHODS

(i) Inference from direct measurement of a commensurately variable factor. The commonest relationship which is used to infer  $Na^+$  concentration in blood plasma is from an estimation of  $Cl^-$ . As can be seen from the block diagrams  $Na^+$  and  $Cl^-$  do not show a constant ratio in all circumstances and so this inference is not valid.

(ii) Inference from the appearance or non-appearance in the urine of a specific substance that it is present in plasma at a value at or above or below threshold level. For this to be useful the renal threshold value for the substance must be a fixed value. This is not the case between individuals and in the one individual. A good example of variation in an individual is the effect of loss of excess  $Na^+$  in relationship to chloride from duodenal fistulae when the kidney excretes chloride from plasma with values well below the normal threshold.

(iii) Inference from a clinical sign that a concomitant change has occurred. Onset of crepitations at the lung base often indicates plethora and dilution of blood proteins but may result from other causes. Peripheral circulatory failure may occur in chloride depletion but this is not the only cause.

From these remarks it will be seen that indirect evidence must be treated with the utmost caution and accepted only when the collected evidence makes the conclusion very probable: e.g. when the conclusion is based on a careful complete clinical assessment of the patient's condition and not upon a sign.

## INTRACELLULAR FLUID

Half of the body weight is due to intracellular water. It is less subject to fluctuation in quantity than is extracellular fluid. The chief cation associated with intracellular fluid is potassium, which influences acid-base balance and osmotic pressure, including the control of passage of water through the cell membrane. Sodium may enter cells and potassium may leave them to a varying but limited degree.

## ELECTROLYTE COMPOSITION OF BLOOD PLASMA

The concentration of electrolytes in the blood plasma may be stated either in terms of milligrams per 100 ml. or, as in the following table, milli-equivalents per litre :

*Blood Plasma*

Cations		Anions	
M. Eq./litre of plasma.		M. Eq./litre of plasma	
Na <sup>++</sup>	144	HCO <sub>3</sub> <sup>-</sup>	27
K <sup>+</sup>	4.5	Cl <sup>-</sup>	103
Ca <sup>++</sup>	4.5	HPO <sub>4</sub> <sup>--</sup>	2
Mg <sup>++</sup>	2	SO <sub>4</sub> <sup>--</sup>	1
		Org. Acids	6
		Protein	16
<hr/> 155.0		<hr/> 155.0	

## APPENDIX

*A Note on the Principles of Flame Photometry*

When a cation is introduced into a sufficiently hot flame it imparts colour to the flame corresponding to one or more characteristic spectral lines. The intensity of this colour varies with the concentration of the introduced cation. These facts are utilized in cationic estimations by flame photometry. A dilute solution of the cation to be estimated is vapourized under constant conditions and introduced into a flame through a special burner assembly. The particular spectral line being investigated is isolated either by a monochromator or a suitable filter and its intensity is measured by means of a photoelectric cell. This

intensity is compared with the intensities derived from a series of standard solutions of known concentrations, and as intensity varies with concentration, the concentration of the cation being investigated in the unknown solution can be determined.

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## CLINICAL BIOCHEMISTRY

### FORMATION OF URINE

#### (1) GLOMERULAR FILTRATION

Cushny put forward the view that all the plasma constituents, excepting colloids of certain complexity, pass through the glomeruli in solution in the same concentration as they occur in the blood stream. He regarded the tubules as entirely absorptive in function. Because of their value to the body such substances as glucose, sodium chloride, water, and other constituents of the glomerular filtrate, are reabsorbed to a varying degree by the tubules, and the remaining fluid and solutes leave the kidneys as urine. In man approximately 1,300 ml. of blood flow through the kidneys per minute, of which about 700 ml. is plasma. In the same time 120 ml. of this plasma is filtered off by the glomeruli. According to Cushny's theory some 170 litres of fluid filter through the glomeruli in 24 hours, of which 168.5 litres would be reabsorbed in the tubules in order to form 1.5 litres of urine of normal concentration. To reabsorb such a large quantity of fluid seems a stupendous task, yet when one remembers that there are nearly one and a quarter million nephrons in each kidney and that the combined length of all the nephrons in the kidneys is approximately 45 miles, the possibility of such reabsorption can readily be admitted. In support of the view of glomerular filtration is the work of Richards and his colleagues. They introduced a fine quartz pipette into Bowman's capsule of the frog (and also of the snake and necturus), collected the filtrate and analysed it by micro-chemical methods. The fluid contained no protein and was of identical composition with the frog's plasma in regard to urea, uric acid, glucose, inorganic phosphate, creatinine and chloride, and also in electrical conductivity and crystalloid osmotic pressure. This and other evidence from similar experiments in mammals is in favour of the capsular fluid being a simple filtrate. It must be remembered when considering renal physiology that the number of glomeruli functioning at any one time varies considerably. Unless there is a special demand for excretion many of the nephrons are temporarily in a resting state but ready to respond to the needs of the body.

## (2) TUBULAR REABSORPTION

The urine of the frog differs markedly from the glomerular filtrate. It contains no glucose, practically no chloride and is acid in reaction (the filtrate is alkaline) and is less in volume. Obviously these changes must have been brought about by the activity of the tubular epithelium which must have absorbed water, glucose and chloride. In the human kidney much of the sodium, chloride, bicarbonate and water are reabsorbed in the proximal tubules and practically all of the glucose. The fluid passing to the distal tubules is isotonic with the body fluids. The final absorption of water probably takes place in the loops of Henle, and of sodium in the distal tubules. Adrenal corticoids increase the reabsorption of Na and Cl ions. The posterior pituitary antidiuretic hormone has a profound effect in facilitating reabsorption of water whereas phloridzin prevents the reabsorption of glucose and causes glycosuria.

Table XII modified from Cushny shows the degree to which the more important components of urine are concentrated by the kidneys.

TABLE XII

Relative Composition of Blood Plasma and Normal Urine in Man

Constituent	Percentage in Blood Plasma	Percentage in Urine	Degree of Concentration
Water	90-93	95-96	—
Protein and other colloids and fats	7-9	—	—
Glucose	0-10	—	—
Na	0-32	0-35	1
Cl	0-37	0-60	1
Ca	0-01	0-015	1-5
Mg	0-0025	0-006	2
K	0-02	0-15	7
Uric Acid	0-002	0-05	25
Urea	0-005	0-15	30
PO <sub>4</sub>	0-003	0-18	60
SO <sub>4</sub>	0-02-0-04	2-0	70
Creatinine	0-001	0-075	75

The constituents of the urine may be divided into:

### NO THRESHOLD SUBSTANCES

These are waste products of no use to the body and so are not reabsorbed in the tubules. Creatinine and  $\text{SO}_4$  are examples of this group and here also urea is usually included, though some 40 per cent is possibly absorbed by diffusion from the tubules into the blood.

### THRESHOLD SUBSTANCES

These include those components which after being filtered through the kidney glomeruli are almost completely reabsorbed in the tubules as they are of special value to the body. Glucose, Na, and Cl are examples of high threshold substances.  $\text{PO}_4$  and K are medium threshold substances. Why urate should not be in the no threshold group is not known, actually some 90 per cent is reabsorbed in the tubules.

TABLE XIII

Approximate Quantity of Certain Constituents of Glomerular Filtrate Reabsorbed by Urinary Tubules in 24 hours

Chloride .. .. 620 grams	Potassium .. .. 32 grams
Sodium .. . . 505 "	Calcium .. . . 17 "
Glucose .. . . 170 "	Phosphoric acid 6.3 "

The osmotic pressure of urine varies considerably but usually is higher than that of blood plasma. The tubular epithelium must perform osmotic work in reabsorbing water from the more concentrated urine. There is a limit to the absorptive power of the tubules corresponding to a concentration of about 1.4 osmoles/L in the urine. This means that a certain minimum amount of water is necessary for any known quantity of solute in the urine. With a mixed diet and a maximal urinary concentration of 1.4 osmoles/L (sp. gr. 1035) the minimum quantity of water excreted is 850 ml. but if the person is fasting this figure may be reduced to 550 ml. The amount of osmotic work performed by the tubules in reabsorbing water is much less if plenty of water is available for excretion. Usually the quantity of urine passed is 1,500 ml. to 2,000 ml. per day.

### (3) TUBULAR EXCRETION

There is convincing evidence that the tubules of certain marine teleosts (which are devoid of glomeruli) can excrete water, creatinine, creatine, urea, uric acid and other constituents of urine, and it is probable that in man the tubules may help to excrete foreign substances introduced into the circulation such as phenol red or complex iodine compounds (e.g., diodrast, used in X-ray urography). However, there is practically no satisfactory support for the view that the tubules can excrete normal products of metabolism in man, with perhaps a doubtful exception in the case of creatinine. In certain experiments in dogs the blood pressure was lowered below the plasma protein osmotic pressure so as to cause cessation of glomerular filtration, and then inulin, phenol red or hippuran was injected intravenously. Inulin was not excreted, but phenol red and hippuran were excreted, apparently by the activity of the tubular epithelium. (Inulin, a polysaccharide with a molecular weight of 5,000 is in normal circumstances excreted by the glomeruli only).

In view of our still imperfect knowledge of the physiology of the kidneys, it is wise from a clinical standpoint to interpret renal function tests as estimations of general renal efficiency without being dogmatic as to the exact nature of the histological structure involved in the pathological process.

## EXAMINATION OF URINE

### (A) COLLECTION AND PRESERVATION OF URINE

For quantitative analyses it is essential that a twenty-four hours' sample of urine be obtained and that the total volume for that time be recorded. At 8 a.m. or other convenient hour the patient completely empties his bladder and the sample is discarded. All specimens are collected until and including that passed at 8 a.m. on the following morning. After thoroughly mixing, the total volume is measured and an aliquot part, e.g., 200 or 300 ml., is used for analytical purposes. For qualitative tests the best time to collect a sample is some two or three hours after a meal when the metabolic products of the food ingested are excreted in maximum quantity in the urine. It is a mistake to use the sample collected before breakfast as such conditions as slight glycosuria or orthostatic albuminuria may not then be

detected. All vessels should be clean and dry, but as a rule it is not necessary to collect the urine under aseptic conditions unless bacteriological investigation is also desired. Storage of the urine in a refrigerator during the collection of the twenty-four hour specimens will retard the development of micro-organisms, but some antiseptic is usually necessary, otherwise urea may be converted into ammonia and  $\text{CO}_2$  by bacteria and glucose fermented by yeast. Acidification of the urine is probably the best method of preserving it. The addition of 1 ml. concentrated hydrochloric acid to every 100 ml. of urine or 60 ml. of 2N sulphuric acid to the twenty-four hours' collection is recommended by Addis and Watanabe. The 60 ml. of acid are placed in a large jar and the samples of urine added immediately they are passed, the contents of the jar being mixed after each addition. By this means the pH of the urine becomes less than 5.0, and micro-organisms fail to grow in such an acid medium. It may be necessary to neutralize the acid before making certain urinary analyses.

### (B) GENERAL CHARACTERS OF URINE

Observations should be made on:

1. Colour.
2. Odour.
3. Specific gravity.
4. Clarity or turbidity.
5. Quantity passed in twenty-four hours.
6. Reaction.

#### (1) COLOUR

The urine is usually amber in colour, but the exact tint varies widely in health due to the degree of concentration and to the reaction. The pigments normally present are urochrome, uroerythrin, uroporphyrin and urobilin. Urochrome is quantitatively the chief pigment in normal urine, but it shows no absorption bands. Uroerythrin is mainly responsible for the deep reddish tinge of urine in acute fevers, and in gross liver disease it may be present in the urine in considerable quantity. It stains amorphous urates red or pink and is the cause of the colour of the "brick dust deposit" that is so frequently seen in urine. Urobilin and the porphyrins are of special clinical significance and are discussed on pp. 43 and 290.

If urine be warmed with strong mineral acids, e.g., hydrochloric acid, it usually becomes more pigmented and turns somewhat red. This is due to the formation of pigments from chromogens, particularly the development of urorosein from its chromogen, which probably is indole acetic acid. Urorosein in amyl alcohol shows an absorption band in the green between the D and E Fraunhofer lines.

Table XIV (modified from Hutchinson and Hunter) indicates the various colours of samples of urine and the cause of the changes in tint:

TABLE XIV

## Causes of Change of Colour in Urine

<i>Colour</i>	<i>Causes</i>
Nearly colourless . . . .	1. Large amount of urine excreted. 2. Diminution of pigment .
Orange coloured . . . . .	1. Small amount of concentrated urine. 2. Increased pigment. 3. Occasionally bile pigment. 4. Pyridium.
Orange coloured to reddish brown . . . . .	1. Administration of rhubarb, senna, chrysophanic acid.
Red . . . . .	1. Blood. 2. Aniline dyes in sweets. 3. Pyridium. 4. Neotropin.
Dark brown . . . . .	1. Methaemoglobinuria. 2. Phenolic drugs.
Port wine . . . . .	Porphyria.
Brownish black . . . . .	1. Melanuria. 2. Marked haemoglobinuria. 3. Alkaptonuria.
Greenish black . . . . .	1. Hydroquinone, carbolic acid, salol, resorcin, naphthalin. 2. Bile pigments.
Yellowish green, green . . . .	1. Bile pigments. 2. Santonin.

- Yellowish and milky . . . . 1. Pus.  
 2. Fat as emulsion.
- Greenish blue, blue . . . . 1. Excess of indigo forming  
 bodies.  
 2. Administration of methylene  
 blue.

Many of the above causes of pigmentation of urine will be referred to again in the text, but it is convenient to consider now the tests for melanogen in urine.

### MELANIN

Melanin is normally found in the choroid of the eye, in the iris, the skin, and as the pigment of the hair.

The enzyme tyrosinase, a copper protein complex catalyzes the oxidation of tyrosine to dopa (dihydroxyphenyl l-alanine) and the oxidation of dopa to melanin.

#### Tyrosinase

Tyrosine  $\longrightarrow$  Dopa  $\longrightarrow$  (Intermediates)  $\longrightarrow$  Melanin.

According to Lerner and Fitzpatrick the reaction of the three basic substances tyrosinase, substrate (tyrosine) and molecular oxygen is controlled by several physico-chemical factors which determine the rate of melanin formation in the epidermal melanoblasts. These factors include catalytic substances, e.g. dopa, which can accelerate the tyrosine-tyrosinase reaction; chemical inhibitors of copper enzymes, e.g. sulphydryl groups, as well as temperature and hydrogen ion concentration. These investigators suggest that in Addison's disease there may be a decrease in concentration of sulphydryl groups at the site of melanin formation. Suntanning as the result of ultra-violet irradiation is associated with increased melanin formation. Ultra-violet radiant energy catalyzes the oxidation of tyrosine to dopa. Small amounts of dopa so formed then catalyze the tyrosine-tyrosinase reaction. Various other effects of ultra-violet radiation also facilitate melanin formation.

Melanogen, the colourless chromogen of melanin, is usually found in the urine of patients in whom there are extensive secondary deposits of melanotic sarcoma in the liver. The fresh urine may be normal in colour, but darkens slowly on standing

owing to oxidation of melanogen to melanin (melanuria). If the urine has not stood for some hours this darkening may not be observed and melanogenuria may not be suspected.

### *Tests for Melanogen in Urine*

#### (1) FERRIC CHLORIDE IN HCl TEST

*Principle.*—Ferric chloride oxidizes melanogen to melanin and hydrochloric acid dissolves ferric phosphate which would otherwise be precipitated.

*Reagent.*—Ferric chloride (10 per cent) in 10 per cent hydrochloric acid.

*Procedure.*—To 5 ml. of urine add about 1 ml. of 10 per cent ferric chloride in hydrochloric acid. The urine may turn brownish black or black.

#### (2) THORMÄHLEN'S TEST

*Principle.*—Melanogen reduces sodium nitroprusside to ferric ferrocyanide (Prussian blue).

*Reagents.*—1. Sodium nitroprusside solution (1 per cent).

2. Sodium hydroxide (40 per cent).

3. Acetic acid (33 per cent).

*Procedure.*—To 5 ml. of urine add 4 drops of sodium nitroprusside solution and 12 drops of sodium hydroxide. Shake well and add sufficient acetic acid to render the mixture acid. If melanogen be present the urine will turn either greenish blue or blue black.

*Clinical value of the Tests for Melanogen.*—Melanogenuria is a rare condition and, as stated above, occurs usually in patients with secondary deposits of melanotic sarcoma, particularly in the liver. As much as 300 grams of pigment have been extracted from a liver containing melanotic growths. In addition to the chemical tests the diagnosis should be confirmed by general examination of the patient. The darkening of the urine on addition of oxidizing agents may occur in a number of clinical conditions, e.g. indicanuria, or if the urine contains an excess of pigment such as urobilin or uroerythrin or when the patient has been taking certain drugs.

#### (2) ODOUR

Normal urine has a characteristic ammonia-like odour.

nuria the odour resembles that of fruit; in intestinal-vesical fistulae, it is faecal; in cystitis or on standing, it may be ammoniacal due to the decomposition of urea with the liberation of ammonia. Various drugs, such as cubebs and turpentine, and some foods such as asparagus, impart their own characteristic odour to the urine.

### (3) SPECIFIC GRAVITY

This may be determined in various ways, but most conveniently by a urinometer. A 24-hour specimen of normal urine has a specific gravity varying between 1,015 and 1,025, but samples taken at random may be far above or below these figures. Following the ingestion of large quantities of water the figure may be 1,003 or less; on the other hand, when fluid is withheld for twenty-four hours, or with excessive perspiration, it may reach 1,030 or even higher. Inability to dilute or concentrate the urine is evidence of renal inefficiency (see pp. 200-2). Urine of low specific gravity may occur in diabetes insipidus, some forms of chronic renal disease and functional nervous disorders, whereas in diabetes mellitus and acute nephritis the figure is usually high. Having measured the amount of urine voided in twenty-four hours an approximate estimate of the total solids may be obtained by multiplying the last two figures of the specific gravity of the specimen at 15° C. by 2.33. The result is the number of grains of solids in one litre of urine, and by simple calculation the amount is estimated for the twenty-four hour excretion.

### (4) CLARITY OR TURBIDITY

Normal urine when passed should be perfectly transparent. Amorphous urates are liable to be precipitated from acid urine as a white or pink deposit, or one simulating brick dust, the colour depending on adsorption of one of the urinary pigments (uroerythrin). Urates are soluble at body temperature, hence the turbidity becomes manifest as the urine cools and the precipitate redissolves on heating.

Oxalates may appear as a glistening layer above a colloidal deposit—sometimes referred to as the "powdered wig."

If the urine be turbid when voided, the appearance may be due to:

1. Amorphous phosphates—readily precipitated in neutral or alkaline urine and are soluble in acetic acid.
2. Carbonates—soluble in acetic acid with effervescence.
3. Pus, which to the naked eye resembles amorphous phosphates, but is readily distinguished microscopically (p. 212).
4. Blood. This may give a red, brown or smoky appearance to the urine and is detected with the microscope, the spectroscope, or by chemical tests. The cells are frequently crenated owing to the osmotic pressure of the urine being usually higher than that of the blood corpuscles, but in hypotonic urines the corpuscles may be swollen.
- 5 Bacteria. These, in large numbers, give a uniform cloud not removed by ordinary filtration. They are readily detected by the microscope. Bacilluria frequently occurs in women, and may be accompanied by symptoms. The cloudiness of decomposing urine is due to bacteria, and when the urine becomes alkaline as the result of ammoniacal fermentation, precipitation of phosphates increases the turbidity.

#### (5) QUANTITY

The total exchange of water per day in man is subject to considerable variation, but the following table indicates average figures for an adult in a temperate climate.

TABLE XV

#### Daily Exchange of Water

<i>Intake</i>			<i>Output</i>
Liquid	1,500 ml.	Urine	1,500 ml.
In food	750 ml.	Skin	600 ml.
From oxidation of		Lungs	400 ml.
food	350 ml.	Faeces	100 ml.
	<hr/>		<hr/>
	2,600 ml.		2,600 ml.

About 60 per cent of the weight of so-called "dry food" in a mixed diet consists of water. Thus a daily diet of about 1,250 grams contains some 750 grams (ml.) water.

The amount of water lost through the renal system is usually balanced by the intake of fluid as such, e.g. water, tea, beverages.

The relative amount of water lost by the excretory mechanisms varies with conditions. During sweating more water is lost

by the skin, in diuresis more by the kidneys, with diarrhoea more by the bowel. The term oxidation in the above table refers to water produced by oxidation in the body of foodstuffs, thus:

100 grams protein oxidized produces 41 grams water.

100 grams fat oxidized produces 108 grams water.

100 grams carbohydrate oxidized produces 55 grams water.

Approximately two-thirds of the body weight is water.

The areolar tissue of the skin and other parts of the body are important storage sites of water. In certain pathological states excess water held in the subcutaneous tissues may be obvious as oedema. After haemorrhage much of the fluid lost from the blood vessels is restored by transfer from the subcutaneous regions and from muscle. Extra-cellular fluid (14,000 ml.) amounts to nearly 20 per cent of body weight or more precisely some 188 ml. per Kg. of body weight. Plasma volume (3,500 ml.) is approximately 45 ml. per Kg. of body weight.

A healthy adult passes on an average about 1,500 ml. (50 ozs.) of urine in twenty-four hours. Relative to their weight, children pass more urine than adults. Under normal conditions more urine is excreted during the day than during the night, but in chronic renal diseases and certain nervous disorders this may be reversed. Exposure to cold, increase in the amount of fluid ingested or nervous excitement causes an increase in the urine excreted. Diminution in the fluid intake or exposure to heat, especially when sweating is profuse, cause lessened urinary output. Polyuria (increased secretion of urine) is frequently found in chronic nephritis, diabetes mellitus, diabetes insipidus (associated with diminished secretion of the anti-diuretic hormone of the posterior pituitary gland), and hysteria. Oliguria (diminished secretion of urine) is common in acute nephritis, in advanced heart disease, fevers, diarrhoea and vomiting. Anuria (complete suppression of urine) may occur in acute nephritis or obstruction of both ureters due to calculi or pressure by tumours. Reflex nervous causes occasionally also give rise to this condition following operations on the urinary tract and also in certain emotional states. Such nervous reflexes probably cause renal cortical ischaemia by contraction of the interlobular arteries just beyond the point of origin of the juxta-medullary glomeruli.

## (6) REACTION

The acidity of the urine may be expressed: (a) in terms of hydrogen ion concentration or (b) as titratable acidity expressed as the number of millilitres of N/10 alkali used for the titration. The pH of urine is determined chiefly by the proportion of dibasic (alkaline) and monobasic (acid) phosphate which it contains. The ratio of these in the plasma is:  $\frac{\text{NaH}_2\text{PO}_4}{\text{Na}_2\text{HPO}_4} = 1/5$ , and in the urine the ratio is usually in the vicinity of 9/1. The kidney receives blood at pH of about 7.40 and forms urine with a pH of approximately 6.0.

The kidneys may excrete daily the equivalent of 40 to 50ml. of normal acid. The reaction of the urine varies from acid to feebly alkaline, depending upon the diet. Fresh urine is usually acid or amphoteric to litmus. During starvation a pH of 5.0 has been recorded. Fevers associated with much protein katabolism, excess of protein in the diet, acidosis, and the administration of various drugs such as sodium or ammonium mandelate, acid sodium phosphate or ammonium nitrate or chloride as well as the ingestion of a ketogenic diet increase the acidity of the urine. The determination of the pH of the urine is important in mandelic acid therapy of urinary infections. An alkaline reaction may be due to a vegetarian diet. It may also occur in the alkaline tide after a meal or in gastric hyperacidity owing to the diversion of the acid from the blood into the gastric secretion. Vomiting causes much loss of HCl from the body resulting in a disturbance of the ionic balance of the blood, thus kations in excess appear in the urine. Sodium and potassium citrate are frequently used in the treatment of pyelitis and they are converted into carbonates in the body. Most of this carbonate appears in the urine and renders it alkaline, whilst some of the carbon dioxide is eliminated by way of the lungs. The administration of alkaline powders in the treatment of peptic ulcer may render the urine intensely alkaline. These powders usually consist of varying proportions of sodium bicarbonate, magnesium carbonate, and calcium carbonate.

*Determination of the pH of Urine*

*Introduction.*—A strongly acid urine may inhibit the growth

or actually destroy micro-organisms which are growing in it. Advantage is taken of this in the ketogenic diet treatment of urinary infections. This treatment is based upon the fact that a patient consuming a diet in which the ratio of fat to carbohydrate and protein is high, e.g. 3:1, excretes acetone bodies in the urine. These include  $\beta$ -hydroxybutyric acid, aceto-acetic acid and acetone. The growth of bacteria is inhibited by 1- $\beta$ -hydroxybutyric acid in concentration of 0.5 per cent or more provided the pH of the urine is low (5.5 or less). If ketosis alone does not render the urine sufficiently acid the patient is given ammonium nitrate which further lowers the pH of the urine and usually enhances the therapeutic effect.

### (1) SILVERTON'S METHOD

*Principle.*—Methyl red in absolute alcohol is added to a measured quantity of urine and the resulting colour matched with standards of known pH concentration, in a comparator.

*Procedure.*—The comparator used is that described by R. J. Silvertton, and consists of three compartments. The colours are estimated by looking through opal glass at a diffused source of light. At each end of the instrument are spaces for three tubes of buffered coloured standards. The pH figures of the six-colour standard solutions are respectively 5.0, 5.2, 5.4, 5.6, 5.8 and 6.0.

To 10 ml. of urine are added five drops of methyl red indicator (0.01 per cent methyl red in absolute alcohol); the colour is distributed by gentle agitation of the tube. The tube of urine is then placed in the comparator and behind it is placed a tube of water. Into the compartment on each side is introduced a colour standard tube and behind each of these is inserted a tube of the patient's urine. The comparison is then made. The colour standards are usually satisfactory for six months provided they are not unduly exposed to light. After this time they should be renewed.

*Clinical value of the test.*—Some patients experience great difficulty in consuming a ketogenic diet which necessarily contains a very considerable amount of fat. A search has been made for some satisfactory substance that could be given by mouth and exert a bactericidal action on the organisms in the urine.  $\beta$ -hydroxybutyric acid is oxidized if administered orally and so

is valueless as a urinary antiseptic when given by mouth. Rosenheim discovered that mandelic acid when ingested was excreted unchanged in the urine, where it had a marked bactericidal action, provided the pH of the urine was in the vicinity of 5.3. This drug is usually administered as sodium or ammonium mandelate and sometimes additional acidifying agents may be necessary to bring the pH to 5.3. Such are acid nitro-hydrochlor (well diluted with water), ammonium chloride and ammonium nitrate. The determination of the pH of the urine is all-important in controlling treatment. Mandelic acid therapy is an extremely useful method of treatment in cases where sulphonamides are contra-indicated or when the organisms are resistant to sulphonamides and other anti-biotics.

## (2) POLYCHROME INDICATOR METHOD

From 5 to 8 drops of indicator are added to 10 ml. of urine in a test tube and comparison of the colour produced with those on the polychrome indicator chart gives an approximate indication of the concentration of hydrogen ions in the urine. This method is sufficiently accurate to be of clinical value.

## CLASSIFICATION OF TESTS OF RENAL EFFICIENCY

Using a variety of tests, the functional response of the kidneys in normal people has been determined by numerous investigators. The mean of the results obtained in each of these tests serves as a standard against which comparison may be made when similar tests are performed on persons judged to be suffering from renal inefficiency.

Blood flows to the kidneys and from it are separated, by these organs, the waste products of the body which are excreted in urine. It is from the examination of these two fluids—blood and urine—that information is obtained which, by inference, gives the clinician some idea of the state of the efficiency of the kidneys.

In investigating the condition of these organs in suspected renal diseases, two methods of approach should be considered: (a) an assessment of renal damage, (b) an assessment of residual renal function.

Whilst evidence may be forthcoming to indicate renal

## IX

### RENAL EFFICIENCY

#### CLASS I. DETECTION OF ABNORMAL CONSTITUENTS OF URINE

##### (1) ALBUMINURIA (PROTEINURIA)

The words "albumin in urine" (albuminuria) are usually used in a loose sense to indicate heat-coagulable protein in urine and this actually includes both plasma albumin and plasma globulin. In normal plasma the protein is approximately 7.5 per cent, albumin being about 5.5 and globulin 2 per cent.

There are two chief views concerning the mechanism of proteinuria.

(1) Protein normally passes in very small quantity into the glomerular fluid and is completely or almost completely reabsorbed by the cells of the tubules. The concentration of protein in the glomerular fluid is probably 25 to 30 mg. per 100 ml. Decrease or absence of reabsorption could then be the cause of proteinuria.

(2) Proteinuria may occur as a result of changes in the glomeruli. Proteins then pass into the glomerular filtrate in excessive amounts and appear in the urine. The presence of abnormal glomerular permeability in the nephrotic syndrome seems to be established. Whether there is in addition any impairment in the tubular reabsorption of protein remains uncertain but Squire and his colleagues have brought evidence suggesting an inability of the tubules to reabsorb protein in patients suffering from this syndrome.

In type II nephritis (Ellis)—which exhibits the nephrotic syndrome—marked albuminuria occurs. The proportion of globulin which accompanies the albumin in albuminuria is usually small and bears no relation to their relative proportions in the blood.

It has been suggested that the reason for the greater abundance of albumin (mol. wt. 67,500) in the urine is due to the fact

that it has a much smaller molecule than globulin (mol. wt. 104,000). As the albuminuria continues the plasma protein falls from the normal of 7.5 per cent to perhaps 4 per cent or less and of this globulin may represent 3 per cent and albumin only 1 per cent.

The recent work of Squire in Birmingham supports the hypothesis that reduced colloid osmotic pressure of the plasma is primarily the cause of the oedema in the nephrotic syndrome. The osmotic pressure of the protein of the blood plasma lies between 25 and 30 mm. Hg. (albumin contributes 80 per cent of this figure). The loss of much albumin in the urine would have a marked effect in lowering this osmotic pressure owing to the fact that, as indicated above, albumin with its smaller molecule and greater percentage concentration in the blood plays a much more important part in maintaining the normal colloid osmotic pressure than does globulin.

A fall of the albumin to below 2.5 per cent with a total protein content of the plasma about 5 per cent is usually the point at which oedema commences, the osmotic pressure of the plasma having fallen by this means to below the critical level of 20 mm. Hg. (For further reference to oedema see p. 174).

*Mucin.*—The delicate transparent cloud deposited by healthy urine contains mucin. Any inflammatory condition of the mucous membrane of the urinary tract may increase the amount of mucin, which goes into solution if the medium is alkaline. It is not coagulated by heat. It is precipitated by acetic acid in the cold, whereas albumin is not. The precipitate is insoluble in excess of acid, whereas the precipitates of euglobulin and nucleo-protein are soluble in excess of this acid and euglobulin is coagulated by heat.

## (2) CAUSES OF ALBUMINURIA

### (1) PHYSIOLOGICAL ALBUMINURIA

It has frequently been observed that the urine passed by adolescents on rising in the morning may be entirely free from albumin, whereas later in the day, much albumin may be present. Owing to the fact that the albuminuria is much more marked when the patient assumes the upright position, the condition is called orthostatic or postural albuminuria. Further-

more, exercise, such as rowing, is prone to induce albuminuria in some persons. The general clinical examination of these patients may show them to be in good health, and their renal efficiency tests reveal no renal disease. However, whilst renal efficiency tests are of value in excluding gross lesions, normal findings with these tests do not necessarily indicate that the kidneys are healthy. Fortunately the patient's subsequent history usually indicates that this type of albuminuria has no pathological significance. Nevertheless, an examination of the centrifuged deposit of the urine should always be made. The presence of red cells and casts should raise the suspicion of organic disease.

## (2) PATHOLOGICAL ALBUMINURIA

There are very many causes of pathological albuminuria but the chief of these may be summarized as:

- |                                   |              |
|-----------------------------------|--------------|
| 1. Acute Type I Nephritis (Ellis) | } Renal      |
| 2. Type II Nephritis (Ellis)      |              |
| 3. Toxaemias of pregnancy         | } Pre-renal  |
| 4. Cardiac failure                |              |
| 5. Febrile conditions             |              |
| 6. Lesions of the urinary tract   | } Post-renal |

The classification of nephritis is a most difficult problem. No ideal classification has been evolved but that of Ellis is of great value in clinical medicine. He divides nephritis into two types:

- (a) Acute Type I Nephritis
- (b) Type II Nephritis.

Davson and Platt in a more recent investigation came to the conclusion that "the Ellis classification is sound in practice and leads in the great majority of cases to clinical and pathological agreement". They point out that the concept so common in text books, of oedematous nephritis as a usual intermediate stage between the acute and chronic phases, is at variance with clinical experience.

### (i) *Acute Type I Nephritis*

This condition is preceded in about 80 per cent of cases by an infection, usually of the tonsils and due to the haemolytic streptococcus. It occurs chiefly in children and young adults.

The capillary endothelium of the glomeruli and the epithelium of Bowman's capsule proliferate and restrict the flow of blood through the glomeruli. It has a sudden onset and is accompanied by albuminuria, haematuria, oedema and a varying degree of hypertension. The volume of urine is diminished. Water and nitrogenous waste products, e.g. urea, are retained. Some 80 per cent recover completely. If the inflammatory process does not quickly resolve, renal ischaemia may cause liberation of excess of renin with subsequent production of hypertensin from hypertensinogen of the blood plasma. This induces a rise in blood pressure and a hypertensive vicious circle is initiated. Death in the acute stage may occur in about 5 per cent from renal failure with uraemia, hypertensive encephalopathy or cardiac failure. In a small group death does not take place for about a year and this condition having an acute onset continues as a subacute nephritis. Some develop chronic type I nephritis (chronic interstitial nephritis, azotaemic nephritis), with azotaemia, rise in blood pressure and finally uraemia and death. Chronic nephritis may last from 5 to 20 years or more.

(ii) *Type II Nephritis (nephrosis, nephrotic syndrome, chronic parenchymatous nephritis, hydraemic nephritis)*

This form of nephritis has an insidious onset and oedema is the outstanding feature and may last for months or years. There is usually gross albuminuria and a marked fall in the plasma albumin but retention of nitrogenous products may not occur for some years. Intercurrent infection is liable to occur in the oedematous tissues but in most instances can be combated by antibiotics. The prognosis of this type of nephritis is not good and only 10 or 15 per cent completely recover. The characteristic glomerular lesion in the oedematous phase is a thickening of the basement membrane of the wall of the capillary loops. Eventually the glomerular capillaries are obliterated and hyalinized and renal ischaemia with resulting hypertension ensues.

(iii) *Toxaemias of pregnancy*

In pregnancy, mild albuminuria occasionally may be present without any other sign of renal impairment or any clinical symptoms. In the more serious condition of pre-eclampsia marked

albuminuria may occur. Although in some cases the blood urea and non-protein nitrogen may be raised above normal, in most of them blood analysis gives little indication of the effect of toxæmia on the kidney. Urea clearance, concentration and excretion tests may reveal varying degrees of less gross renal dysfunction. In the acute toxæmia, eclampsia, nitrogen retention occurs more frequently. Pregnancy may occur in a patient already suffering from chronic nephritis and the blood findings are those of the latter condition (see chapter X). If toxæmia develops during pregnancy in a chronic nephritic patient, the renal damage may be greatly increased.

(iv) *Cardiac failure*

Albuminuria accompanying this condition is due to damage of the renal cells resulting from venous congestion and anoxia and usually diminishes or disappears when the cardiac condition is improved by suitable therapeutic measures.

(v) *Febrile conditions*

A small quantity of albumin may be found in the urine of persons suffering from pyrexia. It is probable that the cloudy swelling of the kidney cells, which may accompany febrile conditions, is responsible for rendering the glomerular epithelium temporarily more permeable to plasma proteins. A few casts may be present, but these and the albuminuria usually disappear as the fever subsides.

(vi) *Lesions of the Urinary Tract*

Lesions of the renal pelvis, bladder, prostate and urethra may lead to proteinuria. Such sources of protein in the urine are not uncommon and the clinician must exclude them before deciding that the proteinuria has a renal or pre-renal causation.

For a fuller discussion of the causes of albuminuria see French, *Index of Differential Diagnosis of Main Symptoms*, 6th ed., Wright, London.

*Tests for Albumin in Urine*

In cases of bacilluria the urine may not filter clear, and in such circumstances a few drops of ten per cent caustic soda should

be added, which precipitates the phosphates, and this precipitate carries down the micro-organisms. On filtering, the filtrate should be reasonably clear, and when neutralized with acetic acid, the tests for albumin may then be performed.

### (1) HEAT AND ACETIC ACID TEST

*Principle.*—Solutions of albumin are coagulated by boiling, and the precipitate is intensified by slight acidification.

*Reagent.*—Acetic acid (2 per cent)

*Procedure.*—A test tube three parts full of urine is held by its lower end whilst the upper portion is boiled. The tube is then viewed against a dark background and any opalescence or cloud in the upper layer is noted. This is usually due to one or more of three things:

- (a) Calcium and magnesium phosphate.
- (b) Calcium carbonate.
- (c) Coagulated protein.

If the urine be alkaline or amphoteric, acidify the upper layer with a few drops of two per cent acetic acid (confirm that it has been made acid by testing with litmus paper) and if the cloud entirely disappears it is due to earthy phosphates. If it disappears entirely but with effervescence, carbonates are present, usually mixed with phosphates. If the cloud is unaltered, or only partially clears, or becomes more flocculent, it indicates the presence of albumin (and globulin). If the urine be very alkaline due to ammoniacal fermentation of the urine or to the patient having taken alkaline powder, then it may be necessary to use 33 per cent acetic acid to render it acid. Bence-Jones' proteins may give a precipitate when the urine is at a temperature of 40 to 60°C but usually redissolve on bringing it to the boil.

*Fallacy.*—There is one fallacy, however, owing to the fact if mucin be present it may be precipitated by the acetic acid, and the precipitate formed be confused with albumin. Mucin is derived from the lower urinary tract or from the vagina and does not indicate renal damage, hence it is most important to distinguish it from albumin. It is not coagulated by heat, but is precipitated by acidification with acetic acid.

If then the heat and acetic acid test shows a precipitate which is judged to be albumin it would be wise to confirm this and

exclude the possibility of the cloud being due to mucin by using the three test tubes method described in the next paragraph.

### (2) THREE TUBES TEST FOR ALBUMIN OR MUCIN

Take three test tubes A, B and C. One quarter fill test tube A with the urine under investigation (filtered if necessary). Into the tube B pour a similar quantity of urine to that in tube A and add sufficient 33 per cent acetic acid to make the contents of this tube markedly acid to litmus paper. A similar quantity of urine is introduced into the third tube C; it is made just acid to litmus and brought to the boil. If a cloud occurs of equal intensity in tubes B and C, then it is due to mucin. If it occurs in tube C only it is due to albumin. Finally, if it occurs in both tubes B and C, but is more dense in tube C, then albumin and mucin are present. Tube A is used as a control.

### (3) SALICYL SULPHONIC ACID TEST

*Principle.*—Albumin forms a white precipitate with salicyl-sulphonic acid.

*Reagent.*—Saturated solution of salicyl-sulphonic acid.

*Procedure.*—Pour about 3 ml. of urine into a test tube and add 6 to 10 drops of saturated aqueous solution of salicyl sulphonic acid. A white precipitate occurs if much albumin be present and a white haze if there is only a small quantity of albumin. A test tube containing urine only should be used as a control. This is an excellent clinical test. The salicyl sulphonic acid test and the heat and acetic acid test are about of equal degree of sensitiveness and approximately four times as sensitive as Heller's test.

*Fallacy.*—Mucin, if present, is precipitated, as is also Bence-Jones' protein but the latter redissolves on warming.

### (3) BENCE-JONES' PROTEINS

These substances, originally thought to be one protein, were first described in 1848 by Bence-Jones and are of rare occurrence. Their molecular weights approximate to that of egg albumin, being about half that of serum albumin. Though Magnus Levy believed them to be of exogenous origin there is now general agreement that they are formed within the body chiefly in the bone marrow. If urine containing Bence-Jones' proteins be

slightly acidified to prevent precipitation of phosphates, and then warmed to between 50° and 60°C, coagulation occurs. On further heating to boiling point, the coagulum redissolves more or less completely. Hopkins has demonstrated that the solution of the coagulum obtained on heating depends on the presence of neutral salts, those with divalent anions as  $K_2SO_4$  acting most effectively in an alkaline medium, and those with divalent cations such as  $CaCl_2$  in an acid solution. The pH of the urine is an important factor in the solution of the coagulum.

It has been shown by Hewitt that Bence-Jones' proteins from various patients suffering from myelomatosis—a disease of bone marrow—differ slightly in their properties. These differences are exhibited particularly in regard to optical rotation, solubility, and amount of salt required to precipitate them. They possess many properties in common with serum proteins, especially pseudo-globulin, but immunological experiments support the chemical findings that Bence-Jones' proteins are distinct from serum albumin and globulin. They are soluble in water and precipitated by about 50 per cent saturation with ammonium sulphate.

The presence of Bence-Jones' proteins in urine almost invariably indicates disease of the bone marrow, usually multiple myeloma and less frequently secondary deposits of malignant disease in bone or possible leukaemia.

### *Heat Test*

Filter the urine if it is not clear and render it faintly acid to litmus. Introduce a few ml. into each of three test tubes. To the second tube add one drop and to the third tube two drops of 33 per cent acetic acid. Put a thermometer in each of the tubes and place them in a water bath. Gradually raise the temperature. Bence-Jones' protein begins to be precipitated between 40° and 60°C, whilst albumin and globulin are not coagulated until above 60°C. On continuing to raise the temperature to boiling point the Bence-Jones' protein redissolves. If albumin also be present it can be filtered off through a hot filter funnel when it is at boiling point. On cooling the filtrate the Bence-Jones' protein precipitates and can be redissolved on heating again to boiling point.

a product of renal epithelial degeneration, and in this matrix may be embedded renal epithelial cells, due to desquamation of the epithelium lining the tubules, red blood corpuscles from the congested blood vessels in the inflamed kidneys, leucocytes, or bacteria; hence the names epithelial casts, blood casts, leucocytic casts and bacterial casts. If the cast consists entirely of structureless material it is referred to as hyaline or waxy, according as it is slightly or highly refractive. Typical hyaline casts are colourless, homogeneous, semi-transparent, cylindrical structures with parallel sides and rounded ends. They are generally straight or less frequently curved and vary greatly in length and breadth. Waxy casts are more opaque than the hyaline type, greyish or colourless, and are usually shorter and broader and have an appearance as if cut from paraffin. All gradations between hyaline and waxy casts occur. If fatty debris (probably derived from some of the formed elements mentioned above) is present in the cast, it is called a fatty cast, or if non-fatty granular material is incorporated it is known as a granular cast. In general, it may be said that epithelial and leucocytic casts indicate acute nephritic conditions; fatty and granular casts tend to occur along with epithelial casts, but, by themselves, indicate less acute conditions than do the epithelial forms. Hyaline casts may be present in all forms of nephritic derangement and even in the absence of renal disease. Blood casts may occur in renal haemorrhage of diverse origin, and by themselves are not evidence of inflammation, but, accompanying other casts, usually indicate acute inflammatory conditions. The amount of albumin and the nature of the casts in urine are not always true criteria of renal disease, and renal efficiency tests should be performed whenever possible to determine more accurately the condition of the kidneys.

## CAUSES OF OEDEMA

### (1) OEDEMA OF ACUTE (TYPE I) NEPHRITIS

In the past this has been regarded as due to a generalized increase in capillary permeability. This can scarcely be the case for it is found that the oedema fluid in nephritis has practically the same protein content as that in congestive cardiac failure and

there is no decrease in blood volume but rather an increase with a fall in haematocrit reading. It is thought that the oedema is due to sodium retention. Such retention of sodium with subsequent oedema is also seen in patients treated with excess of cortisone.

### (2) OEDEMA OF TYPE II NEPHRITIS

In this condition the great loss of protein in the urine causes hypoproteinaemia and hence a fall in the osmotic pressure of the plasma proteins allowing the passage of excess of fluid into the tissue spaces. The oedema of starvation, and of certain liver diseases is chiefly due to hypoproteinaemia although in the conditions mentioned reduction of plasma proteins does not seem to be the entire explanation of the oedema.

### (3) OEDEMA OF CONGESTIVE CARDIAC FAILURE

This is probably of a complex nature. The initial factor may be retention of sodium due to diminished glomerular filtration associated with normal reabsorption of sodium in the tubules. Retention of water follows with increased blood volume and raised venous and capillary pressure. The hydrostatic effect due to this last factor accounts for the distribution of the oedema of cardiac failure.

### (4) INFLAMMATORY OEDEMA

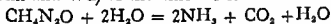
This is associated with increased permeability of capillaries and the passage of protein from the blood plasma into the interstitial fluid so as to cause a disturbance of the normal osmotic equilibrium and retention of excessive amounts of water in the extra cellular fluid. This is seen in cellulitis and other inflammatory conditions.

### (5) LYMPHATIC OEDEMA

This is caused by blockage of lymphatics by tumours and by other mechanical means.

patient's blood should be examined after a twelve hours' fast. The urea is in simple solution and is present in almost equal quantity in corpuscles and in plasma. Even in cases where the kidneys are severely damaged, the amount of urea excreted per day in the urine may be the same as in a normal person, but in such circumstances the concentration of urea in the blood may be much raised, sometimes to as much as 200 mg. or more per 100 ml. The amount of functionally active material in the kidneys is so large that only about one-fourth is actually necessary to carry out the normal renal excretion. It is probable, then, that not until the renal parenchyma is reduced to one-quarter of that normally present will the blood urea vary in proportion to the further damage of the kidney. It must be remembered that only in cases of severe renal damage is the urea content of the blood a useful indication of the state of the kidneys and it is of very little value in lesions of moderate severity.

*Principle.*—The estimation of the amount of urea in blood is based on the conversion of urea into ammonia and  $\text{CO}_2$  by the ferment urease, which is contained in the soya bean, and the subsequent estimation by titration (Maclean) or colorimetrically (Folin and Wu) of the amount of ammonia liberated.



Some of the ammonia will be present in the form of ammonium carbonate, and alkali is added to decompose this.

*Reagents.*—1. Potassium oxalate (20 per cent).

2. Sulphuric acid (5 per cent).

3. Acid potassium phosphate (0.6 per cent).

4. Capryl alcohol.

5. Soya bean meal.

6. Sulphuric acid N/100.

7. Sodium hydroxide N/100.

8. de Wesselow's indicator (p. 357).

9. Solid anhydrous potassium carbonate.

10. Saturated solution of potassium carbonate.

*Procedure.*—About 10 ml. of blood are withdrawn from a vein at the bend of the elbow, coagulation being prevented by the use of a small amount (20 mg.) of finely-powdered potassium oxalate, or two drops of a 20 per cent potassium oxalate solution.

A wash bottle A and two large test tubes B and C about 200 mm. deep and 25 mm. wide with well-fitting rubber stopper are required (see Fig. 23): the tube B need not be of heat-resisting glass as is used in non-protein nitrogen estimation. Through each rubber stopper two glass tubes pass, one of which is long and dips into the liquid contained in the respective test tubes; that in C has a perforated bulb on the lower end. The short tubes pass just through each stopper. The test tubes and wash bottle are connected together by means of tubing having suitable clips E and F attached.

Into the wash bottle A is introduced about 20 ml. of 5 per cent sulphuric acid.

Into B 5 ml. of 0.6 per cent acid potassium phosphate solution are measured, and by means of a special pipette 3 ml. of blood are introduced, and the pipette washed out two or three times with the phosphate mixture. From 6 to 8 drops of capryl alcohol are added and, finally, 0.3 g. of soya bean. Fine grinding of

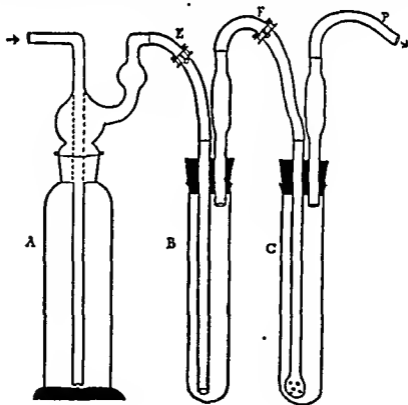


FIG. 23

die during the acute stage of the illness but those with a figure below 100 mg. per cent usually recover and blood urea gradually returns to normal.

If in chronic nephritis the blood urea is above 100 mg. per cent on two successive occasions at an interval of three or four weeks the patient seldom lives for more than two years longer.

Blood urea estimation in polycystic disease of the kidneys or in tuberculosis or tumour of the kidney associated with chronic renal failure usually shows evidence of urea retention.

## (2) EXTRA-RENAL AZOTAEMIA

This may be further divided into: (i) pre-renal and (ii) post-renal.

(i) In *pre-renal azotaemia* much fluid is deviated from the kidney due to such conditions as cardiac failure with oedema, excessive sweating, vomiting or diarrhoea. This means that the glomerular filtration pressure is greatly reduced and there is diminished volume of filtrate with resulting oliguria. A similar diminution of glomerular filtration occurs in very low blood pressure associated with shock. Wood has demonstrated that in severe haemorrhage such as haematemesis the blood urea may rise very considerably, probably due to temporary impairment of renal function caused by the fall in blood pressure and the associated effect of anoxia on the kidney cells. The blood urea usually remains high for one to three days and then slowly falls to normal as the haemoglobin of the blood is restored.

In diabetic coma, if the blood urea exceeds 100 mg. per cent the mortality is high unless by modern therapy the condition is rapidly controlled. Apparently dehydration of the patient, excessive nitrogenous metabolism, impairment of circulation in the kidneys, osmotic changes in the blood plasma and actual toxic damage of the renal tissues are in a varying degree responsible for the rise in blood urea.

The work of Krieger and of other investigators indicates that the normal blood urea in pregnancy is 15-30 mg. per cent. Forty mg. or more of urea per 100 ml. of blood can be regarded as indicating renal inefficiency in this condition. In acute toxæmias such as pre-eclampsia and eclampsia a raised blood urea may be found. Much higher values sometimes occur in patients with pyelonephritis or nephritis complicating pregnancy. When high

blood urea occurs in eclampsia during pregnancy it may persist in the non-pregnant state. There is a very high death rate among chronic nephritic women who develop a toxæmia during pregnancy.

(ii) *Post-renal azotaemia* is usually caused by partial or complete obstruction of the urinary tract by an enlarged prostate, but calculi in the renal tract, congenital abnormality or urethral stricture may be responsible. Unless prolonged obstruction has caused irreversible renal damage successful treatment of the obstruction leads to a restoration of the kidneys to more or less normal. In patients with an enlarged prostate the estimation of blood urea (or non-protein nitrogen), the urea clearance test and the urea concentration test have much bearing on the type of operation to be performed and the desirability or otherwise of preliminary drainage either by in-dwelling catheter or supra-pubic drainage. For unilateral lesions of the kidneys, the indigo carmine test with cystoscopy and the intravenous pyelogram are the two most useful tests.

In making a diagnosis of uraemia it is most important that reliance should not be placed on blood urea determination alone, but it should be confirmed or disproved by other means. If the urea percentage in a sample of urine is two per cent or over, uraemia can usually be excluded.

Finally, it should be remembered that the estimation of blood urea is a measure of the power of the kidneys to excrete nitrogenous substance only at the time of the test. Because of the number of extra-renal conditions which have been seen to affect this function, the urea clearance test or the urea concentration-excretion test (Krieger) usually gives more information concerning renal function than blood urea estimations alone, unless a preliminary estimation of blood urea has shown it to be elevated, when it may be unnecessary or even undesirable to give the patient urea as is done in the Fowweather clearance test or the urea concentration-excretion test (Krieger).

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## XI

### RENAL EFFICIENCY

#### CLASS III. ANALYSIS OF THE URINE

In order that the analytical results may be of clinical value, it is necessary for the patient to be on a standard diet, the composition of which is known. Furthermore, only samples taken from complete twenty-four-hour specimens of urine are satisfactory for analysis. Both of these requirements are difficult to fulfil unless the patient is in hospital, hence the method is not popular in general practice, and the information gained adds little to that obtained by simpler methods of investigation. Table XVII (p. 190) is compiled from various authors.

#### CLASS IV. ESTIMATION OF THE VOLUME OF BLOOD CLEARED OF A KNOWN CONSTITUENT BY THE KIDNEYS IN A GIVEN TIME

##### (1) UREA CLEARANCE

The conception of renal clearance was first introduced by van Slyke in relation to the excretion of urea. It may be defined as the maximum volume of blood which can be completely cleared of the substance in one minute. Actually the kidneys never remove completely the urea from the blood; there is a lower concentration in the renal vein than in the renal artery but urea is never absent from the blood in the renal vein. What we actually measure is "the number of millilitres of blood which contain the amount of urea removed per minute by renal excretion."

Van Slyke states that the chief source of error in his test is incomplete emptying of the bladder and that this factor can be eliminated by calculating clearances on two consecutive hourly samples and averaging the results. Krieger found that the divergence above and below the percentage average normal value in these two calculations was so great in 20 per cent of a series

of tests on over 400 patients that the renal function could not be assessed. Fowweather found similar variations in tests on normal students. He therefore modified the van Slyke method by determining the amount of blood cleared of urea in the second hour after ingestion of 15 g. of urea. The range of values for normal renal function was greatly limited by this procedure. He suggests that the urea acts as a stimulus causing the bladder to completely empty. An alternative explanation is that the diuretic action of the urea causes excretion of a larger volume of urine and thereby greatly reduces the error due to incomplete emptying.

*Blood Urea Clearance Test after Ingestion of Urea*  
(Fowweather Test—modified)

*Principle.*—Two hours after the ingestion of 15 g. of urea an estimate is made of the volume of the blood whose urea content is "cleared" of urea by the kidneys in one minute.

A point of great practical importance is that, by administration of urea and extending the duration of the test to three hours as is adopted in this book, the urea concentration test (Maclean), the total urea excreted in three hours (Krieger) and the urea clearance (Fowweather) are all combined in the one test and the information as would be indicated in Table XIX on page 207, can then be assessed.

It is our practice also to estimate the blood urea of the fasting patient.

*Procedure.*—No breakfast is to be taken on the morning of the test, which is carried out in the following manner:

8.55 a.m.—Blood for urea estimation is taken.

9 a.m.—Patient empties his bladder (exact time to the nearest minute is noted). The urea (15 g. urea in 100 ml. of water) is immediately given.

10 a.m.—Patient empties his bladder (exact time is noted).

10.55 a.m.—Blood for urea estimation is taken.

11 a.m.—Patient empties his bladder (exact time is noted).

12 noon.—Patient empties his bladder (exact time is noted).

All of each specimen of urine passed must be sent to the laboratory. Complete emptying of the bladder is essential and a catheter may be used if necessary. Each specimen is to be

labelled with the name of the patient and the exact time at which it was passed or obtained by catheter.

The above times are given as examples, but the test may be commenced at any suitable time.

The average rise above the fasting level of the blood urea in the second hour following the administration of 15 g. of urea is 20 to 40 mg. urea per 100 ml. of blood.

The specimens of urine and blood may be used for:

- (a) Estimating fasting blood urea.
- (b) Estimating urea concentration in urine (Maclean) (p. 192).
- (c) Estimating the total urea excreted in three hours (Krieger) (p. 195).
- (d) Calculating the urea clearance per minute (Fowweather) (see below).

The clearance is calculated from data obtained from the second specimen of urine and the specimen of blood drawn two hours after the dose of urea.

*Calculation.*—Two modes of urea clearance are recognized: (a) the maximum clearance  $C_m$  which occurs when the flow of urine is over 2 ml. per minute; (b) the standard clearance  $C_s$  when the flow is below 2 ml. per minute.

Maximum clearance  $C_m = \frac{UV}{B}$  = volume of blood cleared of urea in one minute.

Standard clearance  $C_s = \frac{U}{B} \sqrt{V}$

when  $B$  = mg. urea per 100 ml. blood.

$U$  = mg. urea per 100 ml. urine.

$V$  = volume of urine in ml. excreted in one minute.

The normal range for  $C_m$  is 60-95 ml., average = 75 ml.

The normal range for  $C_s$  is 40-65 ml., average = 54 ml.

Expression of the result as "ml. of blood cleared of urea per minute" requires a formula in which the normal average, e.g.,  $C_m$  = 75 ml. and  $C_s$  = 54 ml. are expressed respectively as 100 per cent normal function. All values obtained are then expressed as percentage of the average normal function.

Thus  $C_m = \frac{U \times V}{B} \times \frac{100}{75} = \frac{U \times V}{B} \times 1.33,$

and  $C_s = \frac{U \times \sqrt{V}}{B} \times \frac{100}{54} = \frac{U \times \sqrt{V}}{B} \times 1.85.$

Whichever formula is used, any patient whose test gives 75 per cent average normal function or more is regarded as having reasonably good renal efficiency.

I am indebted to Dr V. Krieger for the accompanying table, which facilitates the calculation of urea clearance. In the above formulae,  $\sqrt{V} \times 1.85$  and  $V \times 1.33$  have been calculated for all volumes, so that the equation becomes

$$\frac{U}{B} \times x, \text{ where "x" is obtained from Table XVI (p. 188).}$$

*Clinical value of the test.*—Harrison quotes van Slyke as suggesting the following interpretation:

<i>Renal function</i>	<i>% of av. normal clearance</i>
Normal	Over 70
Mild deficit	70 to 40
Moderate deficit	40 to 20
Severe deficit	below 20
Uraemic coma present or imminent	below 5

According to Fowweather the blood urea clearance after urea gives a more correct indication of renal function than the clearance test without the previous administration of urea. This view is supported by the researches of Krieger, who has found the Fowweather test of much more value than that of van Slyke.

She agrees that this test alone cannot be used as an accurate guide to prognosis, but must be considered in relation to other tests and to a full clinical review of the patient.

## (2) INULIN CLEARANCE (GLOMERULAR FILTRATION RATE)

There are three ways in which a substance may be eliminated from the body by the kidneys.

- (1) Filtration at the glomeruli only, no absorption or excretion occurring in the tubules.
- (2) Filtration at the glomeruli and excretion by the tubules.
- (3) Filtration at the glomeruli with varying degree of reabsorption by the tubules. This is the usual method.

If a substance is filtered through the renal glomeruli and is neither reabsorbed nor secreted by the tubules it will give a measure of the glomerular filtration rate or, as it is called

TABLE XVI  
FOWWEATHER TABLE

Vol. of urine in ml. per hour		Vol. x		Vol. x		Vol. x		Vol. x		Vol. x		Vol. x		Vol. x		Vol. x		Vol. x		Vol. x	
Vol.	x	Vol.	x	Vol.	x	Vol.	x	Vol.	x	Vol.	x	Vol.	x	Vol.	x	Vol.	x	Vol.	x	Vol.	x
10	0.76	24	1.17	38	1.48	52	1.72	66	1.94	80	2.14	94	2.30	108	2.48	122	2.70	136	3.01	150	3.28
11	0.78	25	1.18	39	1.50	53	1.74	67	1.95	81	2.14	95	2.33	109	2.48	123	2.73	137	3.04	151	3.30
12	0.80	26	1.20	40	1.52	54	1.74	68	1.96	82	2.16	96	2.34	110	2.50	124	2.75	138	3.06	152	3.32
13	0.85	27	1.22	41	1.53	55	1.77	69	1.98	83	2.18	97	2.35	111	2.52	125	2.77	139	3.08	153	3.34
14	0.89	28	1.26	42	1.55	56	1.78	70	2.00	84	2.18	98	2.37	112	2.53	126	2.79	140	3.10	154	3.36
15	0.93	29	1.28	43	1.57	57	1.79	71	2.02	85	2.20	99	2.37	113	2.53	127	2.81	141	3.13	155	3.38
16	0.96	30	1.31	44	1.57	58	1.81	72	2.04	86	2.20	100	2.39	114	2.55	128	2.83	142	3.15	156	3.40
17	0.98	31	1.33	45	1.59	59	1.83	73	2.04	87	2.22	101	2.39	115	2.55	129	2.86	143	3.17	157	3.42
18	1.01	32	1.35	46	1.60	60	1.85	74	2.05	88	2.24	102	2.40	116	2.57	130	2.88	144	3.19	158	3.44
19	1.05	33	1.38	47	1.63	61	1.85	75	2.07	89	2.24	103	2.40	117	2.59	131	2.90	145	3.21	159	3.46
20	1.07	34	1.39	48	1.67	62	1.87	76	2.07	90	2.26	104	2.42	118	2.59	132	2.93	146	3.24	160	3.48
21	1.09	35	1.41	49	1.67	63	1.88	77	2.09	91	2.28	105	2.44	119	2.61	133	2.95	147	3.26	161	3.50
22	1.11	36	1.44	50	1.68	64	1.91	78	2.10	92	2.28	106	2.46	120	2.66	134	2.97	148	3.28	162	3.52
23	1.14	37	1.46	51	1.70	65	1.92	79	2.13	93	2.29	107	2.46	121	2.68	135	2.99	149	3.30	163	3.54

$x = \sqrt{V}$  per min.  $\times 1.85$  for volumes of 10 to 119 ml. per hour

$x = V$  per min.  $\times 1.33$  for volumes of 120 ml. and over per hour

"glomerular clearance". Inulin is thought by many observers to be such a substance as is also the thiosulphate ion. If the volume of urine excreted per minute =  $V$  ml. and the concentration of substance in urine =  $U$  mg./ml., the quantity ( $UV$  mg.) excreted per minute in the urine must equal that produced per minute in the glomerular filtrate. If the concentration in the plasma is  $P$  then  $\frac{UV}{P}$  = plasma clearance which in man is 125 ml. per minute.

Substances whose clearance is greater than the "glomerular clearance" must be in part secreted by the tubules. In man the creatinine/inulin ratio is above unity, whereas for glucose the clearance is zero as it is normally completely reabsorbed by the tubules. It is obvious that in renal disease clearance values may be not only affected by reduction of glomerular filtration rate but also by altered function of the tubules. There may be impairment of tubular secretory function and also a variable degree of back diffusion into the blood plasma of the solutes in the tubular fluid. Normally, urea has a clearance below that of inulin which indicates partial reabsorption of urea in the tubules. There is some evidence that in disease of the kidneys urea may be actually secreted by the tubules.

### (3) DIODRAST CLEARANCE (RENAL PLASMA FLOW)

Diodrast is excreted by the tubules and this leads to a greater clearance than is the case with inulin whose elimination through the kidneys is by the glomeruli only. Para-amino-hippuric acid has a similar method of elimination to diodrast. The determination of the clearance of either of these substances gives an estimate of the renal plasma flow.

Technically it is easier to estimate para-amino-hippuric acid than diodrast and hence the former is generally used for the clearance test (for details of this estimation and that for inulin and thiosulphate, see H. Varley, *Practical Clinical Biochemistry*).

In addition to glomerular filtration rate, and renal plasma flow estimations can also be made of the maximum tubular excretory capacity and the maximum tubular reabsorption capacity. As these methods are somewhat difficult to perform and time-consuming the usual procedure is to determine the urea clearance only.

TABLE XVII  
Blood and Urine Changes in Nephritis

URINE					BLOOD						
	Vol. in ml.	S.G.	Colour	Casts	Albumin	Grms. urea in 24 hrs.	Grms. Chloride in 24 hrs.	Mgm. per 100 ml.			
								N.P.N.	Urea	Uric Acid	Creat- inine
Normal	About 1500	1015- 1025	Amber	Absent or an occasional hyaline	Absent	25-30	10-15	15-40	15-40	1-3	1-2
Acute type I nephritis	500 or less	1020- 1025	Dark red, smoky	Abundant, granular, blood and epithelial forms	Up to 1% or more	8-10	2-0 or less	50-200 or more	50-200 or more	3-8 or more	2-5 or more
Type II nephritis (nephrotic syndrome)	1000 or less	1010- 1030	Variable	All varieties particu- larly fatty and epithelial	Up to 3% or more	10-14	2-0 or less	20-50	20-50	2-5	2-3 or more
Chronic type I nephritis (Azotaemic type)	2000 or more	1003- 1010	Pale	A few hyaline and granular	Trace to 0.5%	12-15	10-15	30-200 or more	30-200 or more	2-10 or more	3-13 or more

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tubing with a levelling tube E. The hypobromite is introduced into the bottle A and the urine into the small tube B. C is a glass tap.

The apparatus (see Fig. 24) should first be tested for leaks by closing the glass tap C and developing pressure by raising the levelling tube. About 10 ml. of sodium hypobromite are introduced into the bottle A and 2 ml. of urine are measured accurately into the small tube B. This tube is now lowered very carefully into the bottle without spilling its contents, the cork is inserted tightly and the bottle is placed in a cold water bath. With the tap C open, the level of water is brought to zero and then the tap C is closed. The urine is mixed with hypobromite by tilting the bottle several times, holding the bottle by the glass parts and not by the stopper. The bottle is replaced in the cold water bath which dissipates the heat evolved in the chemical reaction resulting in the evolution of nitrogen. After a couple of minutes, the menisci of measuring burette and levelling tube are brought exactly level and the volume of nitrogen evolved is read.

*Calculation.*—One gram of urea evolves 354 ml. of nitrogen. If X be the volume in ml. of nitrogen liberated, then

$$\frac{X}{354} \times \frac{100}{2} = \text{percentage of urea in urine.}$$

Table XVIII on page 196 dispenses with the need for calculation. In it the amount of nitrogen evolved in ml. is plotted against the percentage of urea in urine.

*Clinical value of the urea concentration test.*—Normally the maximum concentration of urea exceeds two per cent, and frequently the figure is three or even four per cent. If the percentage is two or less the first thing to exclude as the cause of the low figure is excessive diuresis. The volume of urine in the first hour should not exceed 120 ml., and in the second and third hours not more than 100 ml. In the presence of excessive diuresis low urea concentration is not necessarily an indication of renal inefficiency and the test should be performed again after restriction of fluid for 24 hours. In renal disease, the percentage of urea excreted may be lowered. As stated, figures below two per cent may be taken as indicating renal inadequacy, the degree of lowering of concentration corresponds approximately to the

gravity of the kidney lesion. This test has the advantage of indicating lesions of a slighter degree than are detected by alteration of blood urea; nevertheless, it is a comparatively gross test and a very considerable amount of renal impairment is necessary before it will be discovered by this method. The test is particularly useful in distinguishing uraemia from other conditions having similar clinical features, since uraemia can usually be excluded if the urea content of the urine is 2.0 per cent or more.

It should be noted that in the nephrotic syndrome with oedema but with no nitrogenous retention this test may give normal results, particularly if the output of urine is small, despite the fact that the patient may be desperately ill. On the other hand, the urea may diffuse widely into the oedematous fluid in the various tissues of the body and little is presented to the kidneys for excretion. In these circumstances the percentage of urea in the urine is low.

The test is found to be useful in toxæmias of pregnancy since it is capable of detecting lesions less gross than those indicated by blood urea estimations. If a low urea concentration is followed by a second low value after rest and sedation renal inefficiency is definitely indicated and induction of labour is frequently carried out. Volumes of urine must be observed as well as percentage values. In many toxæmic cases a normal concentration is associated with very small urine volume. In the definitely nephritic types the low urea concentration persists in the non-pregnant period, and a further pregnancy is almost certain to be complicated by toxæmia.

The test is of considerably more value when interpreted in conjunction with the findings for the total urea excreted during the course of the three hours (see Urea Concentration-Excretion Test, Krieger).

#### *Urea Concentration-Excretion Test*

(Krieger's modification of Maclean's method)

*Principle.*—Urea dissolved in water is administered orally and the total quantity of urea excreted in the subsequent three hours is estimated as well as the percentage of urea in each hourly specimen.

*Procedure.*—This is similar to that described in the urea con-

TABLE XVIII

Urea Concentration Table (Percentage)

ml. N<sub>2</sub> liberated from 2 ml. of urine.

	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
2	0.28	0.30	0.31	0.33	0.34	0.35	0.37	0.38	0.40	0.41
3	0.42	0.44	0.45	0.47	0.48	0.49	0.51	0.52	0.54	0.55
4	0.56	0.58	0.59	0.61	0.62	0.64	0.65	0.66	0.68	0.69
5	0.70	0.72	0.73	0.75	0.76	0.78	0.79	0.80	0.82	0.83
6	0.85	0.86	0.88	0.89	0.90	0.92	0.93	0.95	0.96	0.97
7	0.99	1.00	1.02	1.03	1.05	1.06	1.08	1.09	1.10	1.12
8	1.13	1.14	1.16	1.17	1.19	1.20	1.21	1.23	1.24	1.26
9	1.27	1.29	1.30	1.31	1.33	1.34	1.36	1.37	1.38	1.40
10	1.41	1.43	1.44	1.45	1.47	1.48	1.49	1.51	1.52	1.54
11	1.55	1.56	1.58	1.60	1.61	1.62	1.64	1.65	1.66	1.68
12	1.70	1.71	1.72	1.74	1.75	1.77	1.78	1.79	1.80	1.82
13	1.84	1.85	1.86	1.88	1.89	1.91	1.92	1.93	1.95	1.96
14	1.98	1.99	2.00	2.02	2.03	2.05	2.06	2.08	2.09	2.10
15	2.12	2.13	2.14	2.16	2.18	2.19	2.20	2.22	2.23	2.24
16	2.26	2.27	2.29	2.30	2.31	2.33	2.34	2.36	2.37	2.39
17	2.40	2.41	2.43	2.44	2.46	2.47	2.49	2.50	2.51	2.53
18	2.54	2.56	2.57	2.59	2.60	2.61	2.63	2.64	2.66	2.67
19	2.68	2.70	2.71	2.73	2.74	2.75	2.77	2.78	2.80	2.81
20	2.82	2.84	2.85	2.87	2.88	2.90	2.91	2.92	2.94	2.95
21	2.97	2.98	2.99	3.00	3.02	3.03	3.05	3.07	3.08	3.09
22	3.11	3.12	3.14	3.15	3.16	3.18	3.19	3.21	3.22	3.23
23	3.25	3.26	3.28	3.29	3.30	3.32	3.33	3.34	3.36	3.38
24	3.39	3.40	3.42	3.43	3.45	3.46	3.47	3.49	3.50	3.51
25	3.53	3.55	3.56	3.57	3.59	3.60	3.62	3.63	3.64	3.66
26	3.67	3.69	3.70	3.71	3.73	3.74	3.76	3.77	3.79	3.80
27	3.81	3.82	3.84	3.86	3.87	3.88	3.90	3.91	3.93	3.94
28	3.95	3.97	3.98	4.00	4.01	4.02	4.04	4.05	4.07	4.08
29	4.10	4.11	4.12	4.14	4.15	4.17	4.18	4.19	4.21	4.22
30	4.24	4.25	4.27	4.28	4.29	4.30	4.32	4.33	4.35	4.36
31	4.38	4.39	4.41	4.42	4.44	4.45	4.46	4.48	4.49	4.51
32	4.52	4.53	4.55	4.56	4.58	4.59	4.60	4.62	4.63	4.65
33	4.66	4.68	4.69	4.70	4.72	4.73	4.75	4.76	4.77	4.79
34	4.80	4.81	4.83	4.84	4.86	4.87	4.88	4.90	4.91	4.93
35	4.94	4.95	4.96	4.98	4.99	5.01	5.02	5.04	5.05	5.07
36	5.08	5.09	5.11	5.13	5.14	5.15	5.16	5.17	5.19	5.21
37	5.22	5.24	5.25	5.26	5.28	5.30	5.31	5.32	5.33	5.35
38	5.37	5.38	5.40	5.41	5.42	5.43	5.44	5.46	5.47	5.48
39	5.50	5.52	5.53	5.55	5.56	5.57	5.58	5.60	5.61	5.63
40	5.64	5.65	5.67	5.69	5.70	5.72	5.73	5.74	5.76	5.77
41	5.79	5.81	5.82	5.83	5.85	5.86	5.88	5.89	5.90	5.91

centration test of Maclean (p. 192). The data obtained are used for the estimation of the urea excreted in three hours.

*Clinical value of urea concentration-excretion test.*—According to Krieger, who has performed a careful investigation of renal function during the course of pregnancy in a large series of patients, the average excretion of urea was 5.3 g. in three hours in normal women in the last two months of pregnancy. In the earlier months of pregnancy and in the normal non-pregnant woman the average normal excretion was 6.5 g. in three hours. Expressing the average normal as 100 per cent the lower limit of normal was 75 per cent. She has found that the combined figures obtained in this test are of much greater value in interpreting renal function than the percentage concentration figures alone as are used in Maclean's urea concentration test or the total urea excretion alone. The special value then of Krieger's method is that it combines both tests, that is, the concentration and the total excretion in the interpretation of renal function. She has summarized her findings as follows:

- (a) Normal function is indicated when a normal percentage of urea is excreted in normal or large volume of urine so that there is normal total excretion of urea in three hours.
- (b) Renal damage is indicated whenever the total excretion is low:
  - (1) Low percentage of urea excreted in small or normal volume of urine, resulting in low total excretion of urea in three hours.
  - (2) Low percentage of urea excreted in large volume of urine, resulting in low total excretion of urea in three hours.
  - (3) Normal percentage of urea excreted in small volume of urine, resulting in low total excretion of urea in three hours.
  - (4) One percentage normal or high but in small volume and percentage in other two specimens is low so that the total excretion of urea in three hours is low.
- (c) The interpretation is uncertain when the percentage of urea is low and excreted in large volume of urine so that the total excretion of urea is normal or high. The test should be repeated after restriction of fluid for 24 hours.

*Indigo Carmine Test*

*Principle.*—A measured quantity of indigo carmine is injected intravenously and the time of the appearance of the dye at the ureteric orifices is observed by means of the cystoscope.

*Reagent.*—Sterile solution of indigo carmine (0.4 per cent).

*Procedure.*—An intravenous injection of 4 ml. of a 0.4 per cent solution of indigo carmine is given. Using the cystoscope, the dye can be seen issuing from the ureteric orifices in four minutes in normal persons, provided a general anaesthetic has not been given. If the patient be anaesthetized, then the first appearance of the dye may not occur till seven minutes have elapsed.

The administration of one pint of water prior to commencing the test aids in the excretion of the dye.

If the urine is acid or slightly alkaline the indigo carmine appears greenish blue. If the reaction is strongly alkaline the dye may be decolorized. In the presence of marked pyuria the colour may not develop.

*Clinical value of the test.*—Delay in the appearance of the colour, and failure to produce deep coloration are signs of deficient renal function. The test is usually used for the investigation of the function of each kidney separately, the efflux of urine from each ureteric orifice being watched with the cystoscope.

Chromo-cystoscopy is popular among surgeons as it is a rapid method of determining the relative efficiency of the kidneys prior to renal operations.

*Intravenous Urographic Test*

Three groups of iodine containing substances have been used for intravenous urography. These substances have the following common properties:

- (i) they are of low toxicity;
- (ii) they are rapidly excreted by the kidney;
- (iii) they have a relatively high iodine content.

(The high iodine content gives good contrast during X-ray examination.)

The groups are:

- (a) *Iodoxyl*—an iodo-pyridone-dicarboxylic acid compound.

This preparation contains 51.5 per cent iodine and is marketed under various names, such as, Uroselectan B (Schering), Uropac (May and Baker), Pyelectan (Glaxo).

- (b) *Diodone*.—a di-iodo-pyridone-acetic acid compound.

At the moment this group of compounds is the most commonly used as it can be injected into an artery and produces less damage if inadvertently introduced into the tissues around a vein. It contains 49.8 per cent iodine and is marketed under the names of Uriodone (May and Baker), Pyclosil (Glaxo), Diodone (Boots).

- (c) *Amino-iodobenzoic group*—tri-iodo compounds.

These are the most recently developed contrast media and are said to produce fewer reactions in the nature of fluttering, nausea and vomiting in the patient.

Diaglinol (May and Baker) is an example of this group and is sodium acetrizate. It contains 65.8 per cent iodine.

Urografin (Schering) is a similar preparation and contains a mixture of the sodium and methyl-glucamine salts of a tri-iodobenzoic acid compound. It contains 62.1 per cent iodine.

This group of compounds probably holds pride of place as a contrast medium for arteriography and angiocardiology.

The usual adult dose of these various compounds is 20 ml. injected intra-venously.

*General*.—Radiograms are usually made about 6, 15 and 30 minutes after injection, but, if excretion is delayed owing to impaired renal function, examinations may be necessary, one, two or more hours after injection.

In infants, when a suitable vein cannot be found for injection, the contrast medium can be injected intramuscularly, e.g. into both buttocks. The dye is then excreted more slowly and may not outline the renal pelves for 20 minutes after injection.

*Contra-indications*.—Iodine sensitivity, if severe, precludes the use of these substances and a number of deaths has been reported in iodine sensitive patients.

Severe cardio-vascular disease and marked renal insufficiency may also be contra-indications.

### *Concentration Test* (Fishberg)

*Principle.*—The kidney normally has the power of concentrating the waste products of the body in the urine so that the urinary specific gravity (S.G.) is 1,022 or more. Failure to accomplish this concentration is an indication of renal inefficiency.

*Procedure.*—The patient on the evening prior to the test takes his usual meal at about 6 p.m. This should include a generous amount of protein, but not more than 200 ml. of fluid is allowed. Nothing more either of food or fluid is to be taken until the test is completed. The urine passed on retiring and any voided during the night is discarded. On waking in the morning, say, at 7 a.m. urine is passed and kept. The patient remains in bed and an hour later (8 a.m.) passes another specimen. He is then allowed up and about if he is able to be so, and finally passes in another hour's time (9 a.m.) a third specimen. The specific gravity of each of these specimens is determined and under normal conditions should be 1,022 or more.

*Clinical value of the test.*—Fishberg has found this simple concentration test the most generally useful method for studying kidney function. To decide whether there is retention of nitrogenous substances in the blood, the blood chemistry must be studied. As a generalization it may be stated that the lower the specific gravity (below 1,022), the greater is the renal impairment. In Fishberg's experience the kidneys are always able to excrete urine having a specific gravity of at least 1,010 even when there is the greatest of renal damage. If there is reason to suspect very severe renal disease it may not always be wise to do this test, as to refrain from administering fluid to the patient for sixteen or seventeen hours throws an extra strain on the kidneys. In the presence of oedema the test is not reliable as the evacuation of fluid from the oedematous tissues may lower the specific gravity of the urine and give a false impression of impairment of renal function.

In acute nephritis the S.G. of the urine varies, depending upon the urine volume. With oliguria it is usually 1,020 or more. Much ischaemia of the glomeruli occurs with swelling of the endothelium of the glomerular vessels and diminished filtra-

tion. The slower filtration would allow more complete absorption in the tubules, hence the oliguria.

In chronic nephritis (of the azotaemic type) there is polyuria with low S.G., which does not show the variation which occurs under normal conditions. Hayman, Shumway, Dumke and Miller have investigated hyposthenuria (inability to concentrate the urine) in dogs. Hyposthenuria could be produced in these animals by reduction of kidney mass, ureteral obstruction or uranium poisoning. After recovery the animals passed urine of increased volume and decreased S.G. These investigators thought that this was due to increased flow of blood through the remaining nephrons, with an increased volume of glomerular filtrate per nephron, and a "flood diuresis." This view is further supported by the fact that by injection of a concentrated plasma into the blood of such animals or by a lowering of their blood pressure to just above the critical filtration level, the glomerular filtrate was reduced in quantity and the animal excreted a small volume of urine with a relatively high specific gravity due to the more complete absorption of water in the tubules when the glomerular filtrate was smaller in amount. As a result of this and other work Hayman and his associates came to the conclusion that in chronic glomerulo-nephritis and nephro-sclerosis in man the S.G. fell with the decrease in the number of glomeruli until they reached about 750,000 glomeruli per kidney (the normal number being approximately 1,250,000 per kidney) when it became fixed at 1.010 and did not show further diminution despite the reduction in the number of glomeruli. They further demonstrated that the values for creatinine and urea clearance are closely correlated with the number of glomeruli. The specific gravity of the urine may reach a minimum value of about 1.010 whilst the patient may be free from symptoms. With further progress of the renal disease no increased reduction of the S.G. occurs, but increased renal inefficiency may be demonstrated by the clearance tests. They point out that pathological changes may include not only a reduction in area of filtering surface due to diminished number of glomeruli as a result of destruction of many by disease, but decrease in the flow of blood through the remaining glomeruli due to capillary damage within the glomeruli, so that there is less filtrate for a

given filtering area and capillary pressure. It is interesting to note that in their experience if the number of glomeruli was less than 700,000 per kidney the systolic blood pressure was always above 150 mm. Hg.

In experimental nephritis produced by uranium, diffusion of the constituents of the urine into the peritubular capillaries may possibly occur. The specific gravity of an ultra-filtrate of blood plasma is 1.007, so that when the S.G. of urine becomes relatively fixed at 1.010 the tubules have probably lost their power of selectively reabsorbing salts and water and at times may be inert tubes along which a fluid of the nature of an ultra-filtrate of blood plasma passes either to the peritubular capillaries or to the collecting ducts of the kidneys.

### OTHER TESTS OF RENAL EFFICIENCY

Very many other tests have been suggested to determine the efficiency of the kidneys. Those mentioned below have a varying number of advocates and are popular in some laboratories.

#### *Dilution Test*

In disease of the kidneys there may be an inability to excrete dilute urine. In performing this test no fluid is taken after midnight and the bladder is emptied at 7 a.m. followed by the drinking of 1200 ml. of water during the next half hour. The bladder is emptied at 8, 9, 10, and 11 a.m. The volume and specific gravity of each specimen are measured. Practically all the water drunk should have been excreted in this time and at least one specimen have a specific gravity of 1.003 or less. With renal inefficiency, the volumes of urine may be small (less than 100 ml.) and the specific gravity not less than 1.010.

#### *Phenolsulphonephthalein Test*

A measured quantity of the dye is injected intramuscularly and the amount eliminated by the kidneys in one hour and again in a second hour is estimated colorimetrically. It is less sensitive as a test of renal impairment than the concentration tests.

#### *Plasma Chloride*

For details of this estimation see chapter XVIII. Plasma chloride

estimations are of very limited value in renal disease but are of some value as indicating the need to give or restrict salt.

### *Serum Albumin and Globulin*

Details of estimation are discussed in chapter xvii. Serum albumin may be markedly diminished in type II nephritis (Ellis).

### *Blood Uric Acid and Blood Creatinine*

For the method of estimating these substances and their value in determining renal efficiency, see chapter xvii.

### *Serum Cholesterol*

For estimation see chapter xviii. This may be much increased in type II nephritis (Ellis).

## SUMMARY OF THE MORE IMPORTANT QUANTITATIVE TESTS OF RENAL EFFICIENCY

### (1) Estimation of blood urea.

*Normal*—15 to 40 mg. per 100 ml. of blood.

### (2) Urea concentration test (Macleod).

*Normal*—2 per cent or more of urea in urine in second or third hour's excretion.

### (3) Urea clearance test (Fowweather).

*Normal*—70 per cent or more of average normal function.

### (4) Urea concentration-excretion test (Krieger).

*Normal*—6.5 gm. = average excretion of urea in 3 hours.

If the average is expressed as 100 per cent the lower limit of normal is 75 per cent.

## (I) MEDICAL CASES

(i) *Acute type I nephritis (Ellis).*—The course of this disease is best followed by a series of blood urea (or non-protein nitrogen—N.P.N.) estimations at intervals of a week or so. In this way the prognosis can usually be determined with a moderate degree of accuracy. An examination of the urine for blood and casts is also necessary in determining the progress of the disease.

(ii) *Chronic nephritis without oedema (Azotaemic nephritis).*—Blood urea estimations (or non-protein nitrogen), urea clearance tests (Fowweather) and urea concentration-excretion tests (Krieger) are most useful in this condition, chiefly from the

standpoint of prognosis and as a guide to the amount of nitrogenous food that may be allowed.

(iii) *Type II nephritis (Ellis), Chronic nephritis with oedema (Hydraemic nephritis).*—Blood urea estimation is an important guide in determining the advisability in these cases of administering urea therapeutically. Obviously if the blood urea is already raised, urea administration would not act as a diuretic and would have no influence on the degree of oedema. As in chronic nephritis with oedema the blood urea is frequently normal, it might be thought that the estimation is superfluous, but there are so many cases of mixed nephritis in which raised blood urea may be present in an oedematous patient, that a blood urea estimation is necessary. Estimation of the plasma proteins in such cases is of importance before advising a high protein diet.

(iv) *Hyperpiesis.*—Blood urea or N.P.N. estimations, urea clearance (Fowweather) and urea concentration-excretion tests (Krieger) are of considerable value in distinguishing those patients whose hypertension is primarily vascular and whose renal tests are normal from those in whom there is gross renal disease. The prognosis in the latter is much more grave and dietetic restrictions are in general more definitely indicated as uraemia is prone to occur, but cardiac failure, cerebral haemorrhage or coronary occlusion may intervene.

(v) *Uraemia.*—A physician is frequently confronted with an unconscious patient in whom the tentative diagnosis of uraemia has been made. Determination of blood urea and an estimation of the percentage of urea in the urine will usually be sufficient to confirm or exclude the diagnosis. A blood urea of over 100 mg. supports the diagnosis, provided the percentage of urea in the urine is low (under 2 per cent). It is most important that the urine should be examined, as a raised blood urea—suggesting uraemia—may occur in pre-renal deviation as in circulatory failure, in prolonged vomiting, diarrhoea and other conditions in which the renal function may be normal and the percentage of urea in the urine is three or four per cent. Obviously it is impossible to perform the routine urea concentration tests in detail on the unconscious patient. A sample of blood and a single specimen of urine usually obtained by catheterization suffices for the urea estimations.

## (2) SURGICAL CASES

(i) *Enlarged prostate.*—The estimation of blood urea or non-protein nitrogen, the urea concentration test and the urea clearance test have a great bearing on the type of operation to be performed and the desirability or otherwise of preliminary drainage either by indwelling catheter or suprapubic drainage.

(ii) *Unilateral lesions of the kidney.*—Such conditions comprise renal tuberculosis, tumours, calculi, etc. The problems to be decided are: (a) the nature of the lesion; (b) whether it demands nephrectomy; (c) the efficiency of the opposite kidney, if any. At present we are concerned particularly with the third problem. For this purpose the indigo carmine test associated with cystoscopy is very helpful. Intravenous pyelography is also of great value in determining the site of the lesion and the efficiency of the kidneys.

## (3) OBSTETRICAL CASES

Krieger, who has had a wide experience of renal efficiency tests in cases of normal and toxæmic pregnancy, comes to the following conclusions:

The blood urea test is useful in indicating gross renal damage resulting from severe toxæmias of pregnancy or milder toxæmias in association with chronic nephritis during the pregnancy.

The urea concentration-excretion test is a much more sensitive test than blood urea estimation and enables less gross renal damage to be detected during a toxæmic pregnancy. It can also be used to trace the rate of recovery of renal efficiency or prove the presence of permanent damage by observation of the results of tests at intervals for some months after delivery.

The presence of albumin in the urine of a large number of patients whose kidneys had recovered normal function in relation to excretion of urea in tests two months after pregnancy, and the persistence of traces of albumin in the urine of many of these patients even twelve months after delivery show that this is an even more delicate means of indicating that the kidney is not completely normal.

These renal function tests are of great value in the treatment of the current toxæmia. Deterioration in renal function shown

in repeated tests has been found to be an indication of the necessity for termination of a pregnancy, even though the clinical condition of the patient has temporarily improved as the result of treatment. If investigations are carried on over a period of months after the initial toxæmia, useful information for the management of subsequent pregnancies is gained.

A study of the urea concentration-excretion during and after pregnancy shows that the excretion of urea by the kidney is decreased slightly even in normal pregnancy, and that marked diminution in renal efficiency frequently occurs in severe toxæmia during pregnancy. Deterioration in renal function shown in repeated tests has been found to be an indication that there is a grave risk of degenerative changes in the kidney becoming irreversible unless the pregnancy is terminated; this is true even though the clinical condition of the patient has improved as the result of treatment.

Recovery of normal function often takes place within eight days and usually within two months of delivery by which time maximum urea excretion is attained. In eclampsia the recovery is slower, the maximum excretory power being attained only after a period of twelve months. Persistence of poor renal function tests for twelve months or more after a toxæmic pregnancy indicates a chronic renal condition which will make a further pregnancy dangerous if toxæmia should again develop.

Krieger further found that an important relationship exists between the urea concentration-excretion tests and the Fowweather tests, these tests agreeing in nearly 70 per cent of the patients examined. Marked variations in volume and urea excretion in each of the three hours following ingestion of urea have a profound effect on the Fowweather value and account in most instances for the discrepancy when it occurs between the two tests. When a low urea concentration-excretion test occurred in conjunction with a normal or high Fowweather value the former test was of greater significance. When the initial blood urea was high, a low Fowweather test frequently occurred in association with a normal urea concentration-excretion test and in such cases the Fowweather test represented the more accurate measure of renal efficiency. When a normal urea concentration-excretion test occurred with a low Fowweather test, which could

not be accounted for because of high blood urea, no evidence was available to indicate which test was correct. If poor renal efficiency has been demonstrated in one pregnancy chemical tests should be carried out in any subsequent pregnancy and attention paid to these tests even though the pregnancy, clinically, appears normal.

It is useful to express the findings of renal efficiency tests in tabular form, shown in Table XIX.

TABLE XIX  
Report on Renal Efficiency

1. BLOOD UREA	Fasting_____
	Two hours after 15 gm. urea_____
2. UREA CONCENTRATION TEST	1st hour_____ % Vol._____
	2nd hour_____ % Vol._____
	3rd hour_____ % Vol._____
3. EXCRETION OF UREA IN THREE HOURS	_____ gm.
4. UREA CLEARANCE TEST (FOWWEATHER)	_____ % Average normal function
5. QUALITATIVE TESTS	Albumin_____
	Blood _____
6. MICROSCOPIC EXAMINATION OF URINARY SEDIMENT	Casts_____
	Red blood corpuscles_____
	Pus _____
	Micro-organisms _____
	Crystals _____

## INTERPRETATION OF REPORT ON RENAL EFFICIENCY

### (1) QUANTITATIVE TESTS

Discussing first the quantitative tests, assessment of renal efficiency may be made by the consideration of the following observations:

- (a) fasting blood urea;
- (b) blood urea two hours after 15 g. of urea;
- (c) urea concentration test (Maclean);
- (d) urea concentration-excretion test;
- (e) Fowweather clearance test.

The conclusions enumerated below seem justified.

1. If all of the above tests are normal, renal efficiency is indicated.
2. High blood urea, poor concentrating power, low total excretion of urea, low Fowweather test, all indicate renal inefficiency.
3. One may find high blood urea and low Fowweather test coupled with normal concentrating power and normal total excretion of urea. Here the high blood urea and low Fowweather indicate renal inefficiency, but the other tests normal renal function. Provided there is no doubt of the chemical work, the high blood urea and Fowweather test, indicating renal inefficiency, would more likely be correct. The reason for this explanation is that a high head of urea in the blood may force even a damaged kidney to excrete a greater amount of urea than it normally would do.
4. The Fowweather test is often fallacious when the amount of urea excreted per hour varies markedly. In most normal persons, approximately the same amount of urea is excreted in each hour of urea concentration test. If "peak" or "dip" curves occur, the Fowweather test is frequently at variance with the total excretion of urea. The latter has been found in such cases to fit the clinical picture more satisfactorily.
5. The total excretion of urea is unreliable when a poor concentrating power is coupled with excessive excretion of urine so that the total excretion of urea is normal. Repetition after restriction of fluids for 24 hours before the test may give results which can be readily interpreted. If the result is similar to the first one, it is likely that the kidney being tested is severely damaged and is trying to compensate by secreting large volumes of dilute urine.

## (2) QUALITATIVE TESTS

The significance of the presence of albumin in urine can only be determined by considering the findings of the general clinical examination of the patient. These findings may, for example, indicate acute nephritis and the albuminuria is then one piece of evidence in demonstrating severe inflammation of the kidneys. On the other hand, the clinical examination may reveal congestive cardiac failure; then, albumin in the urine is due to passive congestion of the kidneys and is not a sign of severe kidney damage. It will almost certainly disappear, leaving unimpaired kidneys, when the circulation is restored by suitable therapeutic measures. The more insight one has into the clinical background of the patient's illness, the greater is the likelihood of giving a true interpretation of the significance of albuminuria. Blood in urine may or may not be due to renal disorder. For further discussion of haematuria, see page 283.

## (3) MICROSCOPIC EXAMINATION OF URINARY SEDIMENT

The significance of casts in the urine is discussed on page 173 and of red blood corpuscles on pages 283-4.

Pus in urine (pyuria) is not necessarily due to renal involvement (pyelitis, pyelonephritis), but occurs in association with suppuration in any part of the urinary tract or when pus in adjacent areas gains access to the urinary tract. Micro-organisms, e.g. tubercle bacilli, may indicate disease of the kidneys and hence some degree of renal inefficiency. Crystals (calcium oxalate, sulphonamide, etc.) in the urine suggest the possibility of a calculus in the kidney and renal impairment, although such crystalluria may be transient, due to ingestion of foods (e.g. rhubarb or spinach) or of a drug of the sulphonamide series.

## REFERENCES

Part I—nephritis. *Q. J. Med.*, 18, 149.

### XIII

## URINARY SEDIMENTS

The microscopic examination of urinary sediments may give valuable clinical information, and in some diseases such as pyelitis and bacilluria it is the chief evidence upon which a diagnosis is made. The urine should be centrifuged, or, if a centrifuge is not available, allowed to sediment in a tall glass cylinder and the deposit examined microscopically, first under the low and then under the high power. If kept for longer than six hours a preservative should be added (see p. 151).

The sediments of the urine may be of two types:

"Organized" sediments; (Fig. 25).

"Unorganized" sediments.

### ORGANIZED SEDIMENTS

1. *Casts*.—These have been described in chapter ix.

2. *Epithelial Cells*.—These are derived from the mucous membrane of the urinary tract and may be divided into the following groups:

(a) Small, round, or polyhedral cells, about the size of a pus corpuscle or a little larger and containing a single round nucleus. They may come from the deep layers of any part of the urinary tract and may be found embedded in casts, where they usually show varying numbers of fat droplets.

(b) Transitional cells, pear-shaped, spindle-shaped, round, or having a tail-like process. They are chiefly derived from the transitional epithelium, which lines the bladder, ureters and pelvis of the kidneys.

(c) Squamous cells, which are large, flat cells derived from the superficial layers of the urethra and bladder. In non-catheter specimens of urine obtained from women, squamous vaginal epithelia are frequently present.

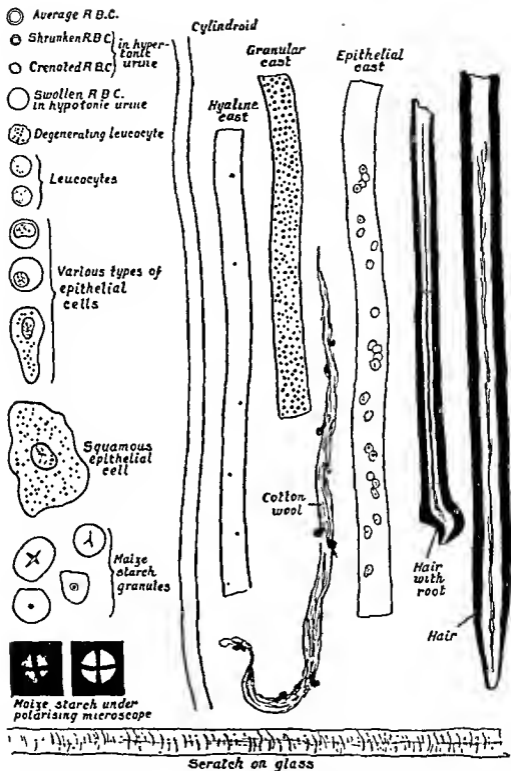


FIG. 25. Diagram to assist in the identification of cells, casts and starch granules. All are drawn to the same scale using a micrometer eye-piece. (Harrison, *Chemical Methods in Clinical Medicine*)

3. *Leucocytes*.—A few leucocytes may be present in normal urine, but become very numerous as a result of suppuration in the urinary tract. In these circumstances, micro-organisms should be carefully looked for and cultures of the urine made. When at all abundant, pus forms a precipitate, which is distinguished from other precipitates by microscopic examination. The granules of the pus cells may be clearly demonstrated by running a little dilute acetic acid under the cover slip. In alkaline urine, the pus corpuscles are often markedly degenerated, and are swollen, transparent and with little granular structure. Pus cells may be distinguished from epithelial cells of a similar appearance by the fact that they stain mahogany brown with a solution of iodine in potassium iodide, whereas this reagent stains the epithelial cells a light yellow.

4. *Erythrocytes*.—These cells may be normal, swollen or crenated, depending upon the osmotic pressure of the urine. They occur in the condition known as haematuria, which is discussed in detail in chapter xvii.

5. *Animal parasites*.—There may be present ova of bilharzia, cysts, hooklets and shreds of membranes of echinococci, and embryos of filaria. Other animal parasites may be found in the urine in tropical countries.

6. *Spermatozoa*.—These are recognized by their characteristic structure.

7. *Micro-organisms*.—Normal urine, as formed by the kidney, contains no micro-organisms. It is free from bacteria while in the bladder, but may be slightly contaminated when passing through the urethra. The commonest organism found pathologically is the bacillus coli, but many other organisms may be present, including tubercle or typhoid bacilli. It is frequently desirable to obtain a catheter specimen of urine for bacteriological examination. Catheterization should always be performed with rigid aseptic precautions. Full details of the bacteriological investigation of urine will be found in textbooks of bacteriology.

8. *Prostatic threads*.—These may occur in chronic inflammation of the prostate gland, and are usually microscopic in

size and of such density that they usually fall to the bottom of the glass cylinder.

9. *Foreign substances*.—Contamination of the urine may occur, due to starch granules, yeast cells, hyphae or spores of fungi, fibres of wool, cotton or linen, pollen of flowers kept in the patient's room, and particles of food or hair.

Of these, starch granules are the most frequent foreign substances found in the urine, particularly of infants, being derived from dusting powders. They stain blue with iodine and hence are readily recognized.

### UNORGANIZED SEDIMENTS

- |  |   |
|--|---|
| A. Those which may be found in acid urine include:     | { <ol style="list-style-type: none"> <li>1. Uric acid</li> <li>2. Sodium urate</li> <li>3. Amorphous urate</li> <li>4. Calcium oxalate</li> <li>5. Cystine</li> <li>6. Tyrosine</li> <li>7. Leucine</li> <li>8. Bilirubin</li> <li>9. Sulphonamide derivatives</li> </ol> |
| B. Those which may be found in alkaline urine include: | { <ol style="list-style-type: none"> <li>1. Calcium phosphate</li> <li>2. Ammonium magnesium phosphate</li> <li>3. Ammonium urate</li> <li>4. Carbonates</li> </ol>   |

This classification is useful, but is not absolutely accurate, since the characteristic sediments of acid urine may remain after the urine has become faintly alkaline, while some of the alkaline sediments may be precipitated in a urine which is still slightly acid.

#### (A) ACID URINE

1. *Uric acid*.—Uric acid may appear in the urine in the following forms:

- (a) Wedge-shaped crystals
- (b) Dumb-bells
- (c) Rhombic prisms

- (d) Whetstones
- (e) Rosettes
- (f) Plates

The crystals are usually coloured brownish red, forming a deposit which may adhere to the sides and bottom of a vessel containing urine, but they may be perfectly colourless. Their presence does not necessarily mean that the uric acid content of the urine is increased, as the deposit may simply be due to increased acidity of the urine. They are soluble in sodium hydroxide. A deposit of uric acid crystals has little clinical significance, unless it occurs before or soon after the urine is voided, since every urine if kept acid will in time deposit uric acid. Their presence in freshly voided urine in clusters may suggest a renal, ureteral or vesical calculus, especially if blood is also present.

2. *Sodium urate*.—The crystals of this salt consist of spheres possessing numerous spines radiating from the surface. They somewhat resemble the "thorn apple" crystals of ammonium urate, but they are not so deeply pigmented.

3. *Amorphous urates*.—These are urates of sodium and potassium. They are usually red or pink in colour, forming a "brick dust" deposit, but may appear white or cream, and resemble a deposit of phosphates, from which they may be distinguished:

- (a) by their solubility on warming to about 40°C;
- (b) by their insolubility in acetic acid.

This deposit occurs frequently in febrile conditions, and has a fine granular appearance under the microscope.

4. *Calcium oxalate*.—Although usually in acid urine, calcium oxalate crystals may also be found in urines which are alkaline. They frequently occur after the ingestion of foods containing much oxalate, such as rhubarb, spinach and strawberries, and also in digestive disturbances associated with fermentation of carbohydrate. Two forms may be observed:

- (a) the envelope form (octahedral);
- (b) the dumb-bell form.

They are extremely hard and readily form calculi.

Calcium oxalate is insoluble in acetic acid, but is soluble in hydrochloric acid, whereas uric acid and urates are insoluble in hydrochloric acid.

5. *Cystine*.—Cystinuria is due to obscure abnormality of protein metabolism and usually continues throughout life. Cystine, which is a sulphur-containing amino acid, is deposited in urine as transparent, colourless, hexagonal plates soluble in ammonia. It may be distinguished from hexagonal crystals of uric acid by adding a drop of hydrochloric acid, which dissolves the cystine but not the uric acid. Its insolubility in acetic acid distinguishes it from phosphates. Furthermore, when heated on platinum foil it burns with a bluish-green flame. If the urine is alkaline it should be acidified with acetic acid and allowed to stand for an hour and then centrifuged. If this is not done the cystine may not be deposited.

In addition to identifying cystine by its microscopical appearance a useful test is to fill a test tube half full of urine and boil with 3 ml. of 40 per cent sodium hydroxide. This liberates sulphur and on the addition of a few drops of lead acetate a black precipitate of lead sulphide occurs. This test is not conclusive if the urine contains protein as this would give the test for sulphur. Paper chromatography is the quickest method of detecting cystine.

6. *Tyrosine*.—Tyrosine is an amino acid containing a hydroxyaromatic group which gives a red colour with Millon's reagent. In acute yellow atrophy of the liver, acute phosphorus poisoning and occasionally in cirrhosis of the liver, tyrosine may appear in the urine and is usually associated with leucine. It forms colourless sheaves of fine needle-like crystals, soluble in ammonia and hydrochloric acid, but not acetic acid, acetone, alcohol or ether. The crystals are not infrequently stained yellow owing to jaundice and choluria which may accompany the diseases in which tyrosine appears in urine. These crystals must not be confused with stellar phosphates, uric acid or bilirubin crystals. Remember that phosphates are soluble in acetic acid, bilirubin in acetone, and that uric acid is not soluble in hydrochloric acid which dissolves tyrosine. It is also readily detected by paper chromatography.

7. *Leucine*.—In acute yellow atrophy of the liver, crystals of this amino acid may appear in the urine in the form of yellow spherical masses, soluble in acid and alkali, but not soluble in ether, and so distinguished from globules of fat. They must not be confused with ammonium urate crystals, which are darker in colour, have superficial spicules, give the murexide test and are insoluble in acetic or hydrochloric acid. The presence of leucine may be confirmed by paper chromatography.

8. *Bilirubin*.—An acid urine containing much bile may show a deposit of bilirubin either in the form of brown needles, rhombic plates or amorphous material. It may stain other crystalline and amorphous deposits. Bilirubin dissolves readily in acetone.

9. *Sulphonamide derivatives*.—Comparatively insoluble acetyl derivatives are formed in the body from sulphapyridine, sulphathiazole, sulphadiazine, and other sulphonamides, and may be precipitated in the urine in characteristic crystalline form. Colloids in the urine apparently have an influence in determining the specific morphology of the crystalline structure. The concentration of sulphapyridine, sulphathiazole and sulphadiazine and their acetyl derivatives in the urine of patients treated with these compounds can exceed the solubility of the same drugs in water and normal urine. This may be explained by the formation of some colloidal chemical complex. The excess of colloid may be derived from damage of the renal tract caused by the drugs in question. Lehr and Antopol have given an excellent account of the various crystalline forms.

(a) *Acetyl sulphapyridine*.—These crystals may be in the form of "whetstones," transparent and colourless and usually with serration of their edges. "Arrowheads" are also common, which are wedge shaped with rounded base and serrations on the sides. "Sheaves" may be present and aggregate to form crosses and starlike crystals.

(b) *Acetyl sulphathiazole*.—Dumb-bell and rosette forms are the usual types. They are amber-green in colour, and marked radial striations occur. Hexagonal platelets, also amber-green, may be present.

(c) *Sulphadiazine and acetyl sulphadiazine*.—Free sulphadia-

zinc is present as green globules which may have needle-like processes. "Shocks of wheat with eccentric binding" is the chief form in which acetyl sulphadiazine crystallizes.

(d) *Acetyl sulphaguanidine*.—Simple rectangular oblong plates with slight bulging in the long axis are seen and are usually as clear as glass, or at times may have a mesh-like pattern. They may aggregate to form stars or cross-like masses. Free sulphaguanidine is five times as soluble as the acetylated derivative and seldom appears as crystals in the urine.

## (B) ALKALINE URINE

1. *Calcium phosphate*.—Inorganic phosphate occurs in the urine as salts of sodium, potassium, calcium, and magnesium, and its combination with sodium and with calcium far exceeds quantitatively its corresponding combinations with potassium and with magnesium.

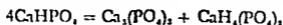
Phosphoric acid, being trivalent, forms three classes of salts, which may be represented as:

$\text{NaH}_2\text{PO}_4$	Monosodium phosphate (acid sodium phosphate)
$\text{Na}_2\text{HPO}_4$	Disodium phosphate
$\text{Na}_3\text{PO}_4$	Trisodium phosphate
$\text{CaH}_2(\text{PO}_4)_2$	Monocalcium phosphate
$\text{CaHPO}_4$	Dicalcium phosphate
$\text{Ca}_3(\text{PO}_4)_2$	Tricalcium phosphate.

The monosodium phosphate and disodium phosphate are very soluble in water and are never found precipitated in urine. Trisodium phosphate is not a constituent of urine.

The calcium and magnesium phosphates occur in less quantity in urine than do the phosphates of sodium and potassium, the ratio ranging from 1:2 to 1:4. Monocalcium phosphate is the most soluble of the phosphates of calcium; dicalcium phosphate is much less soluble and tricalcium is practically insoluble.

If a urine, the reaction of which is close to neutrality, be heated, dicalcium phosphate is decomposed and tricalcium phosphate may be precipitated:

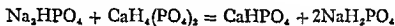


If a urine be made alkaline, the calcium and magnesium salts are precipitated.

Two forms of calcium phosphate precipitate occur in urine:

(a) Amorphous phosphates (earthy phosphate of calcium and to a less extent of magnesium) increase on heating and are soluble in acetic acid. They form a flocculent white deposit.

(b) Stellar phosphates (monohydric calcium phosphate —  $\text{CaHPO}_4$ ). These consist of colourless prismatic crystals occurring singly or in radiating clusters. Occasionally they may appear as needles resembling tyrosine crystals or as thin, irregular, colourless plates. They may appear in faintly acid as well as in alkaline urine, excess of disodium phosphate causing their precipitation according to the following equation:



2. *Ammonium magnesium phosphate.* This phosphate, known as "triple phosphate," occurs in urine which has undergone ammoniacal fermentation. The crystals are colourless, imperfect prisms which vary considerably in appearance and are described as:

- (a) "knife rests";
- (b) "coffin lids";
- (c) "feathery stars".

They are all soluble in acetic acid (2 per cent).

3. *Ammonium urate.*—This is frequently present in urine in cases of cystitis. The usual form is that of small, dark, spherical masses covered with spines — characteristic "thorn apple" crystals. Occasionally it appears as dumb-bells or sheaves of fine needles.

4. *Carbonates.*—When a patient has taken carbonates therapeutically or is on a diet largely vegetable in origin, carbonates may be present in abundance in the urine. They usually occur as amorphous granular particles or in dumb-bell forms, soluble in acetic acid with effervescence.

The following table from G. A. Harrison's *Chemical Methods in Clinical Medicine* summarizes the solubility of the chief deposits which may be found in urine.

TABLE XX

Solubility of Chief Deposits which may be found in Urine

Urinary Deposit	Solubility in					
	<i>Alkalis</i>	<i>Dilute Mineral Acid</i>	<i>Acetic Acid</i>	<i>Alcohol</i>	<i>Acetone</i>	<i>Ether</i>
Uric acid and urates	Sol.	Insol.	Insol.	Insol.	Insol.	Insol.
Earthy phosphates	Insol.	Sol.	Sol.	Insol.	Insol.	Insol.
Calcium oxalate	Insol.	Sol.	Insol.	Insol.	Insol.	Insol.
Cystine	Sol.	Sol.	Insol.	Insol.	Insol.	Insol.
Tyrosine	Sol.	Sol.	Insol.	Insol.	Insol.	Insol.
Leucine	Sol.	Sol.	Sol.	Slightly Sol.	Insol.	Insol.
Bilirubin	Sol.	Sol.	Sol.	Slightly Sol.	Sol.	Slightly Sol.

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amide derivatives. *Am. J.*

Ann. 400, 12, 200.

## XIV

### CALCULI

#### URINARY CALCULI

##### (A) THE FORMATION OF URINARY CALCULI

Normal urine contains a large variety of crystalloids, many of which are ionizable salts, whilst others are non-ionizable compounds such as urea and creatinine. The calculus-forming substances are chiefly calcium oxalate, uric acid and calcium phosphate.

Some of these salts, such as oxalates, are present in urine in a concentration which far exceeds the amount which would be possible if dissolved in water only; in fact, four times as much may be present as is required to produce a saturated solution. There must be some mechanism for preventing their precipitation from the supersaturated urine. This is probably accomplished by adsorption of the oxalates and other salts on to colloids which are present in the urine. The normal urinary colloids include mucin, nucleic acid, chondroitin sulphuric acid and glycogen, which together amount to about 0.8 g. of colloid per litre.

The amount of calculus-forming salts which can be held in solution depends on the surface area of the colloid, and therefore on its state of subdivision. It is probable that the precipitation of these salts is primarily dependent on the aggregation of the hydrophile colloid particles to form large masses, which have relatively less adsorbing surfaces for the crystalloids, and so they are precipitated, usually as calcium oxalate, phosphate, or uric acid crystals. What actually causes this primary aggregation of colloid is not known with certainty, but it may be associated with the neutralization of their surface ionic charges. Fortunately the colloid present in the urine is of the emulsoid type, and is not readily precipitated by small variation in the electrolytes present in the urine, which would be the case if the colloids were suspensoids. If the latter were present, calculus formation would be much more common.

A crystalline deposit, once having been formed, tends to grow by aggregation of further crystals, and thus forms a true primary calculus. According to Joly, the development of the secondary from a primary calculus depends upon the irritation of the wall of the urinary tract by the primary calculus which causes a reactionary exudate to occur. This colloidal exudate of protein material may, by coating the calculus, render it temporarily non-irritating and the exudation will cease. A further precipitation of crystals on the calculus will once more initiate an irritative phase associated with more colloidal exudate. By alternation of crystalloid and colloid, a laminated secondary calculus is formed. It is to be noted that a primary calculus is formed of masses of urinary crystals irregularly arranged, without concentric lamination, and with little colloid present. A secondary calculus has laminae of compact concentrically arranged crystalline material alternating with softer layers containing considerable amount of colloid. If the various crystalline laminae differ in their chemical constituents, the calculus is described as compound, but if the crystalline material is similar throughout it is termed a simple calculus. The majority of urinary calculi are mixtures (compound) and not "pure" stones. A stone originally formed in the kidney may migrate along the ureter to the bladder and further growth in size may occur in this new situation. The usual site of calculus formation in the kidneys is in the lower calyx.

Other factors which must be considered in relation to calculus formation are:

- (a) reaction of the urine,
- (b) urinary stasis,
- (c) oliguria,
- (d) diet,
- (e) certain diseases,
- (f) some drugs,
- (g) infection in urinary tract.

(a) A markedly acid urine will accentuate the tendency for precipitation of certain constituents such as uric acid and calcium oxalate, whilst an alkaline urine will induce phosphate deposition. Owing to the reaction of urine varying from time to

time it is very frequently found that a calculus may be a mixture of phosphate and calcium oxalate, the former being precipitated in an alkaline urine and the latter when the reaction is acid.

(b) The rate of flow of a fluid with crystals in suspension largely determines the tendency of such crystals to precipitate; the slower the current, the greater the liability of deposition. The relative stasis that occurs in the lower calyx of the kidney is probably responsible for this being the commonest site for primary calculus formation.

(c) Oliguria or diminished secretion of urine results in the production of a highly concentrated urine, thus increasing the possibility of precipitation of constituents which are normally held in solution.

(d) The consumption of certain articles of diet rich in oxalates is frequently responsible for oxaluria. Examples of such foods are rhubarb, spinach and strawberries. After ingestion of these foods the concentration of oxalates in the urine may be so increased that crystallization of calcium oxalate may occur, with all the possibilities of calculus formation. A diet containing much purine may be responsible for the appearance of uric acid crystals in the urine, whilst a vegetable diet may cause deposition of phosphates owing to the fact that such a diet renders the urine alkaline. In animal experiments a diet deficient in vitamin A has caused increased calculus formation whilst administration of excess of vitamin D may induce hypercalcaemia and production of calculi.

(e) In certain diseases, notably acute yellow atrophy of the liver and parathyroid adenoma, excess of metabolic constituents may be excreted. In acute yellow atrophy, leucine and tyrosine may form urinary calculi. In parathyroid adenoma hypercalcaemia occurs and excess of calcium in the urine may be responsible for calculus formation.

(f) Some of the sulphonamides, e.g. sulphathiazole, sulphadiazine, may form crystalline precipitates in the urinary tract unless liberal quantities of fluid are ingested.

(g) The preceding account of calculus formation refers only

to the process as it occurs in sterile urine. Very frequently the urine is infected, and calculus formation in such circumstances exhibits certain features not present when the urine is sterile. In the first place in infected urine there is available pathological cellular debris which may act as a nucleus on which the crystalline material may be deposited. Such a nucleus may consist of epithelial cells, leucocytes, or micro-organisms. Furthermore there is abundance of binding material present in the form of colloid derived from the inflammatory exudate associated with infection; such binding material may play a very important part in causing aggregation of the crystals. The calculi in such circumstances usually contain more colloid and are more friable than when formed in a sterile environment. With inflammation in the wall of the urinary tract there is also greater tendency to urinary stasis than when no infection is present. It must be remembered that though the early growth of a calculus may take place in a sterile urinary tract, its future development may occur in an infected medium teeming with micro-organisms and rich in inflammatory products.

Calculi in the kidneys are usually composed of calcium oxalate, or calcium oxalate and phosphate together, or they may be phosphatic calculi in relatively "pure" form. In the urinary bladder the calculi are usually not "pure" but may consist of phosphate admixed with either urate or oxalate, but occasionally they may consist of phosphate alone. The liberation of ammonia from urea in an infected urine renders the urine alkaline and thus phosphates are deposited. X-ray examination will usually reveal calcium oxalate or phosphatic calculi but uric acid stones are not opaque to X-ray.

The urine of a patient suspected of having a renal or vesical calculus should always be carefully examined microscopically for crystals, red blood corpuscles and pus cells. Its reaction should be noted and cultures made to determine the presence of bacterial infection. Cystoscopy, catheterization of the ureters and pyelography, either ascending or descending, may be necessary to obtain full information concerning the calculus and its situation. The indigo carmine test should be performed, or if the patient is given 15 g. of urea in 100 ml. of water about one hour before the ureteric catheters are passed samples may be

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collected from each kidney and examined for the percentage of urea. These last two tests are of considerable importance in determining the efficiency of the individual kidneys when nephrectomy is being contemplated.

## (B) TYPES OF CALCULI

### (1) URIC ACID AND URATE CALCULI

Uric acid and also ammonium urate constitute a considerable proportion of urinary concretions. These stones are hard and vary in colour from pale yellow to brownish red. They both give the murexide test, and ammonium urate evolves ammonia on boiling with sodium hydroxide.

### (2) CALCIUM OXALATE CALCULI

These calculi occur in two forms:

- (a) a small smooth stone frequently referred to as the "hemp-seed" calculus;
- (b) a medium or large, uneven surface stone called the "mulberry" calculus.

These calculi are intensely hard and readily cause haemorrhage. They are soluble in hydrochloric acid without effervescence, but are insoluble in acetic acid. On heating they are converted into calcium carbonate, which dissolves in acetic acid with effervescence.

### (3) PHOSPHATE CALCULI

These stones consist of calcium phosphates and/or, triple phosphate, and generally contain some calcium oxalate and ammonium urate. They are somewhat variable in colour, being grey, white or yellow and have an uneven surface. They do not burn when heated and are soluble in acetic acid without effervescence. On heating with potassium hydroxide ammonia is liberated from calculi containing triple phosphate.

### (4) CALCIUM CARBONATE CALCULI

These concretions are very rare in man, but frequently occur in herbivora. They are usually small, white or greyish calculi, spherical in form, with a hard smooth surface. They are soluble in acid with the evolution of carbon dioxide.

## (5) XANTHINE CALCULI

These calculi occur very rarely. They vary in colour from white to reddish brown and may be mixed with uric acid or urates. They assume a wax-like appearance on rubbing, and on heating they char without a flame. When heated with nitric acid they give a yellow colour which becomes red when potassium hydroxide is added.

## (6) CYSTINE CALCULI

Cystinuria is associated with an inborn error of metabolism, whereby cystine fails to be oxidized. The crystals which appear in urine may aggregate to form a calculus, usually small, oval or cylindrical, white or yellow in colour and somewhat soft in consistency. On heating they burn with a blue flame.

## (7) UROSTEALITH CALCULI

These stones are extremely rare. When moist they are soft and elastic, but when dry they become brittle. They consist of fatty acid and cholesterol. They burn with a luminous flame.

## (8) FIBRIN CALCULI

Fibrin is formed from fibrinogen in the process of coagulation of blood. If this occurs in the urinary tract a mass of fibrin may accumulate and form a fibrin calculus, or more frequently act as the nucleus of other forms of calculi.

*Analysis of Urinary Calculi*

Note the *appearance* and *consistency* of the stone and whether homogeneous in composition, and then crush the whole calculus or a portion of it until it is in a condition of fine powder. If the calculus is markedly laminated, it is wise to saw through the nucleus and separate the various layers and analyse these separately.

*Heat a portion of the powdered calculus on platinum foil.* The substance may leave:

(1) practically no ash;

(2) considerable ash;

(1) *Calculus leaving practically no ash on heating may contain one or more of the following:*

(a) *Ammonium urate*.—This evolves ammonia on heating the original substance with strong potassium hydroxide, and also gives the murexide test. To perform the murexide test a small portion of the powdered calculus is placed in a porcelain basin and two or three drops of strong nitric acid added and the whole evaporated to dryness on a water bath. On now adding a little ammonia to the dry residue, a rich purple colour develops if uric acid or urates be present. A drop of strong potassium hydroxide on another portion indicates the presence of uric acid or urates by the development of a blue colour.

(b) *Uric acid*.—When heated, uric acid chars without a flame, and it gives a typical murexide test.

(c) *Xanthine*.—When heated, xanthine chars without a flame, and it gives with potassium hydroxide a red colour changing to purple red on heating.

(d) *Cystine*.—Cystine is crystalline, soft, pliable, soluble in ammonia, and this solution on evaporation develops crystalline hexagons of cystine. Heating on platinum foil gives a blue flame and an odour of sulphur dioxide.

(e) *Urostealith*.—This gives, on heating, a yellow flame with the odour of burnt shellac or benzoin. It is soluble in ether and alcohol.

(f) *Protein material*.—This consists of serous exudate induced by irritation of the developing calculus or inflammatory products in an infected urine and traces of fibrin.

(2) *A calculus leaving a considerable ash on heating may contain one or more of the following:*

(a) *Potassium or sodium urate*.—The ash is soluble in water and the original substance gives the murexide test.

(b) *Magnesium or calcium urate*.—The ash is not soluble in water and the original substance gives the murexide test.

(c) *Calcium carbonate or magnesium carbonate*.—The ash is soluble in acetic acid, with effervescence.

(d) *Calcium oxalate*.—The ash is soluble in acetic acid, with effervescence, for heat has converted oxalate into carbonate.

(e) *Earthy phosphate*.—The ash is soluble in acetic acid, without effervescence.

For clinical purposes the scheme of analysis shown in Table XXI, page 228, is useful.

## BILIARY CALCULI

### (A) FUNCTIONS OF THE GALL BLADDER

To understand the formation of gall stones, some knowledge of the functions of the gall bladder is essential. These may be conveniently considered under the headings of:

- (1) storage and evacuation;
- (2) absorption;
- (3) secretion.

#### (1) STORAGE AND EVACUATION

During fasting the tone of the sphincter of Oddi increases so that it can resist a pressure of about 300 mm. of bile. The secretion from the liver continues even during fasting, and when the pressure reaches about 70 mm. bile it passes up the cystic duct past the valves of Heister and into the gall bladder. During digestion the sphincter of Oddi partially relaxes and bile may now flow into the intestine when the pressure is 100 mm. bile or so. From time to time the gall bladder contracts, raising the pressure perhaps to 200 mm. and gushes of bile enter intermittently into the duodenum. The chief factor in causing emptying of the gall bladder is fat in the food, e.g. cream, butter, egg-yolk. According to Ivy these are responsible for the liberation of a hormone "cholecystokinin" from the mucosa of the small intestine which is then conveyed in the blood and causes contraction of the gall bladder. It is well known that persons suffering from cholecystitis avoid fats as they tend to make their symptoms worse. Adrenaline or sympathetic stimulation is stated to cause contraction of the gall bladder.

#### (2) ABSORPTION

During its stay in the gall bladder, bile becomes more concentrated owing to the absorption of water and salts through the gall bladder wall and the addition to the bile of mucin from the biliary mucosa. The biliary constituents may vary independently of one another. In this way the bile of the gall bladder may be concentrated eight to tenfold as compared with that obtained from a biliary fistula; hence 50 ml. of stored gall

bladder wall by micro-organisms is probably the cause of the formation of biliary calculi in most instances. Recent work has indicated that in human bile cholesterol is in the form of complexes with bile salts. Such complexes are water soluble. If these are subjected to dialysis the bile salts dialyze away and cholesterol is precipitated. The normal gall bladder mucosa absorbs water, bile salts and cholesterol at a certain rate and in certain relative proportions. Now it has been shown experimentally that the infected gall bladder mucosa absorbs bile salts more readily than cholesterol and the latter tends to be precipitated to form calculi just as we noted it was precipitated in the dialyzing experiments quoted above. In some cases the irritation of gall stones already formed in a sterile gall bladder may lead to secondary bacterial infection and cholecystitis.

### (3) HYPERCHOLESTEROLAEMIA

Aschoff and his school hold that disturbances of cholesterol metabolism associated with hypercholesterolaemia are factors in the formation of gall stones, and this view is supported by many other observers. Moynihan found increased blood cholesterol in a large percentage of his patients who were suffering from gall stones. He regards biliary infection and hypercholesterolaemia as the two chief factors in gall stone formation. Other observers have not found an increase in blood cholesterol in patients with gall stones. Increased blood cholesterol may occur from ingested cholesterol in food, by inefficient excretion due to hepatic disease or possibly by synthesis in the body. Excess of cholesterol in the bile may be due to (a) Increased secretion of cholesterol by the cells of the intra-hepatic biliary tract, (b) Disintegration of the cholesterol containing cells lining the gall bladder, (c) Excretion of cholesterol by the intact mucosa of the gall bladder, and (d) Deficient absorption of cholesterol by the gall bladder wall. Sufficient accurate data are not available to enable one to be dogmatic concerning the relative roles played by these several factors.

### (C) TYPES OF BILIARY CALCULI

#### (1) CHOLESTEROL CALCULI

Cholesterol is a secondary alcohol belonging to the terpene

group, and is classed as a sterol. It is soluble in fat solvents, such as chloroform and ether. Large amounts of bile salts and an alkaline medium both favour its solution. In bile it is in the colloidal state forming an emulsoid, and may be precipitated by alteration of its electric charge. Cholesterol calculi are usually faceted, have a soft nucleus, a stratified outer covering and contain a varying amount of bile pigment. Some cholesterol stones which are not laminated and which have a marked crystalline structure may contain almost 100 per cent of cholesterol.

### (2) BILIRUBIN CALCIUM CALCULI

These stones are usually smaller than the cholesterol stones. They contain calcium bilirubinate mixed with mucus and are usually brownish black in colour and moderately hard.

### (3) CALCIUM CARBONATE AND CALCIUM PHOSPHATE CALCULI

These, though common in herbivora, are extremely rare in man.

## (D) RESULTS OF COMPLETE OBSTRUCTION OF THE COMMON BILE DUCT

### (1) IMPAIRMENT OF LIVER FUNCTION

When the intra-hepatic pressure of bile rises to 300 mm. further secretion ceases. If the obstruction is maintained for many weeks the hepatic cells become atrophied and stained with bile pigment. The bile canaliculi are dilated. A fall in plasma protein concentration gradually occurs. Quantitative tests of hepatic efficiency may show impairment of function.

### (2) GENERAL EFFECTS ON THE TISSUES OF THE BODY

Loss of appetite, nausea and vomiting may ensue. The patient becomes jaundiced and bile pigments and bile salts appear in the urine.

### (3) DIGESTIVE DISTURBANCES

Fat is normally converted into fatty acid and glycerol by the action of lipase. The fatty acids are probably combined with bile salts to form a water soluble complex which is absorbed and phosphorylated in the intestinal mucous membrane. In the

absence of bile from the intestine, hydrolysis of the fat may occur to a considerable extent but the fatty acids are not absorbed and are excreted in the faeces, which are pale in colour owing to the absence of bile pigment from the intestine and the excess of fat and fatty acids. The fat-soluble vitamins are not absorbed and in the case of vitamin K this leads to diminution in the formation of prothrombin with subsequent deficiency in the coagulability of the blood and tendency to haemorrhage. Night blindness of obstructive jaundice is due to vitamin A deficiency.

### *Analysis of Biliary Calculi*

Grind the calculus in a mortar with 10 ml. of ether. Filter and preserve filtrate (A) and residue (B).

*Filtrate (A).*—To the filtrate add an equal volume of 95 per cent alcohol and evaporate on a water bath. When concentrated, take a drop of the solution and place on a microscope slide, and allow to evaporate spontaneously. Now examine for cholesterol crystals, which appear as characteristic four-sided plates with a notched or re-entrant angle. Continue the evaporation of the main solution, and when completed perform the following test for cholesterol:

*Liebermann-Burchard test.*—Dissolve a little of the residue from the evaporated filtrate in 2 ml. of chloroform in a dry test tube. Add 10 drops of acetic anhydride and 1 to 3 drops of concentrated sulphuric acid. If the solution becomes red, then blue, and finally bluish-green in colour, it indicates the presence of cholesterol.

*Residue (B).*—Extract the residue with dilute hydrochloric acid and filter. Test a portion of the filtrate for calcium by adding ammonium oxalate, and another portion for phosphorus by the ammonium molybdate test. Wash the residue (on filter paper) with a little water. Dry the residue on filter paper, then treat it with 5 ml. of chloroform, filter, and test the filtrate for bilirubin. Extract the residue with 5 ml. of hot alcohol. If biliverdin be present, it gives a green colour to the alcoholic extract.

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## XV

# ENDOCRINE GLANDS

## INTRODUCTION

The hormones are produced by the ductless (endocrine) glands of the body and their functions seem to be to determine certain physiological processes and the rate at which these and the related biochemical reactions proceed. The cortex of the adrenal glands is a major factor in controlling the level of sodium and potassium in the blood plasma and the metabolism of carbohydrate, fat and protein. Excess of the principle of the parathyroid glands causes hypercalcaemia, whereas the reverse is the case in parathyroid insufficiency—a state leading to disturbances in muscular activity. The pancreas contains two principles, insulin and glucagon, which, by their prime effects on carbohydrate metabolism, profoundly influence the chemical composition of the body; while the gonadal hormones play important roles in development and in the modification of sex characteristics. The pituitary gland, of two well-defined lobes, secretes a number of principles, of which some eight have been unequivocally identified. These either influence physiological mechanisms *per se* (vasopressin and oxytocin from the posterior lobe and probably growth hormone and fat-mobilizing factor from the anterior lobe), or function by controlling the activity of other glands: thyrotrophin (TSH), the three gonadotrophins, and adrenocorticotrophin (ACTH). In the latter instances a certain measure of reciprocal control exists. Thus, an increased output of the thyroid hormone, with marked effects on metabolic rate, leads normally to a diminution in the production of thyrotrophin by the pituitary gland.

The emphasis in recent years on the dynamic state of the body's constituents has led in an increasing degree to the implication of the hormones in many metabolic disorders. Methods have been devised whereby deviation from the normal levels of function of the glands can be determined with sufficient accuracy

to be of value to the clinician. With the close link between metabolism and hormones the measurement of a particular constituent of the body fluids can frequently indicate the nature of the endocrine lesion (electrolytes, glucose). The assay of circulating or urinary hormones or their degradation products is usually time-consuming and requires particular techniques and skill, but much progress is being made along these lines. The determination of plasma protein bound iodine (P.B.I.) or urinary 17-ketosteroids or corticoids or the gonadotrophin of pregnancy urine are cases in point. Much more difficult are the assays of plasma insulin or, say, thyrotrophin.

### THE THYROID GLAND

The thyroid gland largely consists of vesicles or acini, normally containing colloid, the chief component of which is the glyco protein thyroglobulin. This on hydrolysis yields in addition to other amino acids and sugars, the iodine-containing amino acids thyroxine, mono- and di-iodotyrosine, with a smaller amount of tri-iodothyronine. Although these iodo-compounds have also been shown to exist free in minute quantities in thyroid tissue, only thyroxine (and possibly tri-iodothyronine) represents the active form of the thyroid hormone in circulation. Thyroglobulin is normally restricted to the thyroid tissue, but it may be detected among the plasma proteins following destructive bombardment of the gland with large doses of radioactive iodine ( $^{131}\text{I}$ ). Certain similarities in the structures of thyroxine and di-iodotyrosine

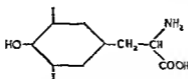


FIG. 26. Di-iodotyrosine

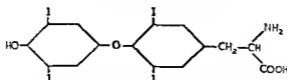


FIG. 27. Thyroxine (tri-iodothyronine has one iodine atom missing adjacent to the hydroxyl group)

(depicted above) suggest that the latter substance is the immediate precursor of thyroxine, a conversion which can be made to occur to a significant extent on warming slightly alkaline solutions of di-iodotyrosine. However, the iodination of tyrosine and the subsequent condensation reaction to give thyroxine may take place *in vivo* within a protein or polypeptide chain. Artificial thyroproteins may readily be produced by careful iodination of iodine-free proteins, e.g. casein and serum albumin. Such thyroproteins yield thyroxine (and di-iodotyrosine) on hydrolysis and have been shown to be effective in clinical hypothyroidism.

The average normal human thyroid gland weighs 20-25 g. and contains 8-10 mg. of iodine of which about 3 mg. are present as thyroxine. The rate at which thyroxine is released to the blood from its stored form as thyroglobulin depends mainly upon the level of thyrotrophin stimulation and the quantity of iodine available for resynthesis by the gland. Excessive amounts of iodide on the other hand may inhibit the release of thyroxine by the gland; this can be easily demonstrated experimentally by the use of  $^{131}\text{I}$ , the turnover of which by the thyroid is depressed by concomitant administration of iodide. In clinical hyperthyroidism this effect of iodide can be used to produce, at least temporarily, an amelioration of the symptoms. The approximate daily requirements of iodine are given in Table XXIII.

In Fig. 28 the dynamic aspects of iodine metabolism are shown diagrammatically. Iodide is rapidly absorbed, mainly through the intestine. The level at which it circulates is dependent upon two main factors: loss through excretion by the kidney, and trapping by the thyroid gland. The efficiency of the latter process greatly exceeds that of any other organ in the body and it is usually further increased in the hyperthyroid state. The trapping mechanism can be greatly depressed by thiocyanate or perchlorate (iodate). Within the thyroid gland the iodide is rapidly bound to tyrosine (probably in peptide or protein form by a system of oxidizing enzymes) and thereby converted into mono- and di-iodotyrosine and thyroxine and tri-iodo-thyronine, and stored in these forms as thyroglobulin. The organic-binding reactions are inhibited by thiouracil and related drugs. Release from thyroglobulin is probably effected by proteolytic enzymes, but as stated previously the only organic form of iodine normally

TABLE XXIII  
Recommended Daily Dietary Allowances, 1954\*

Subjects	Age (years)	Calories	Protein (g)	Vit. A (I.U.)	Thiamine (mg.)	Riboflavin (mg.)	Niacin (mg.)	Ascorbic acid (mg.)	Vit. D (I.U.)	Calcium (g)	+Iron (mg.) <sup>§</sup>	+Iodine (µg)
Men	25	3,000	65	5,000	1.5	1.6	15	30	—	0.8	12	100
	35	2,900	65	5,000	1.5	1.6	15	30	—	0.8	12	100
	45	2,800	65	5,000	1.4	1.6	14	30	—	0.8	12	100
	65	2,500	65	5,000	1.3	1.6	13	30	—	0.8	12	100
Women	25	2,200	55	5,000	1.1	1.4	11	30	—	0.8	12	100
	35	2,100	55	5,000	1.1	1.4	11	30	—	0.8	12	100
	45	2,000	55	5,000	1.0	1.4	10	30	—	0.8	12	100
	65	1,800	55	5,000	0.9	1.4	9	30	—	0.8	12	100
Pregnant (third trimester)	—	Add 400	80	6,000	1.3	1.8	13	80	400	1.5	15	150
Lactating (850 ml. daily)	—	Add 1,000	100	8,000	1.6	2.0	16	100	400	2.0	15	150
Infants	Under 1	60-45/lb.	1.6/lb.	1,500	0.5	0.9	5	30	400	0.6-1.0	6.5	—
Children	1 to 4	1,300	40	3,000	0.7	1.0	7	30	400	1.0	7.5	150
	5 to 8	1,700	55	3,000	0.9	1.3	9	30	400	1.0	10.5	150
	9 to 12	2,300	70	3,000	1.2	1.7	12	30	400	1.2	13.5	150
Boys	13 to 15	3,000	85	5,000	1.5	1.8	15	50	400	1.4	13.5	150
	16 to 19	3,600	100	5,000	1.8	1.8	18	50	400	1.4	15.0	150
Girls	13 to 15	2,500	80	5,000	1.3	1.8	13	50	400	1.4	13.5	150
	16 to 19	2,300	75	5,000	1.2	1.8	12	50	400	1.4	15.0	150

\* These allowances are recommended for the planning of practical diets for healthy persons, normally vigorous, and living in Australia in a warm temperate climate. The recommendations for infants refer to diets consisting mainly of cow's milk; the values do not necessarily apply to breast-fed infants. Ref.: *Med. J. Aust. II*: 3, p. 113, 17 July 1954.

† Columns marked thus are taken from the *Report of the Committee on Nutrition (1950)*, British Medical Association.

§ Normal healthy persons should obtain an adequate intake of iron from a mixed diet containing a variety of foods; those suffering from anaemia related to an iron deficiency will require supplements of iron in medicinal form. (Comment in article in *Med. J. Aust.* referred to above)

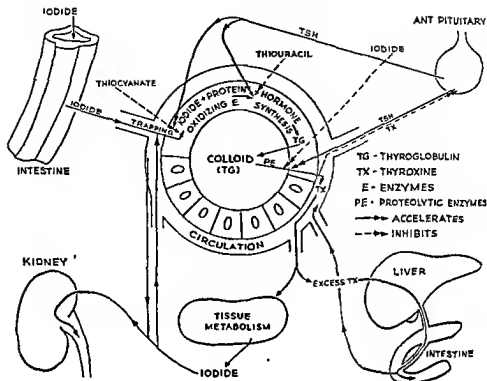


FIG. 28

present in the blood is thyroxine (and possibly small amounts of tri-iodothyronine). Thyroxine is degraded in the tissues mainly to iodide; minor amounts have been shown, in <sup>131</sup>I studies, to be diverted as the glucuronide via the liver, gall bladder and intestine, part being lost to the faeces and part reabsorbed as thyroxine.

According to Riggs the normal human thyroid gland secretes about 70  $\mu\text{g}$ . thyroxine per day, the total amount of extra-thyroidal thyroxine being calculated as approximately 1.2 mg. The level of the plasma protein-bound iodine (PBI), which is almost entirely thyroxine, loosely associated with protein, provides the most reliable chemical index of the state of activity of the thyroid gland. Its estimation, however, requires special skill and experience. Winikoff discusses the results obtained in Melbourne with 106 normal individuals and 137 patients suspected of thyroid disease. The mean normal value was 5.13  $\mu\text{g}/100\text{ ml}$ . (range 2.7-8.0  $\mu\text{g}$ .). In the hyperthyroid patients the mean value was 10.2  $\mu\text{g}/100\text{ ml}$ . (range 7.5-20.6  $\mu\text{g}$ .). When all patients were taken into account a 90 per cent agreement with clinical appraisal

was achieved, a higher correlation than was obtained on the same patients by Clarke and Aujard using the physical index of  $^{131}\text{I}$  uptake by the thyroid gland. Providing suitable counting equipment is available, a procedure involving the  $^{131}\text{I}$  tracer technique has the advantage of simplicity.

The accuracy of the chemical (PBI) and physical ( $^{131}\text{I}$ ) methods is dependent among other factors on the previous medication and dietary history of the patient. Thus iodine in any form or recent administration of an anti-thyroid drug (such as thiouracil) may give erroneous results.

In addition to the two methods just mentioned and observations of the clinical signs of hyperthyroidism or hypothyroidism, estimation of the sleeping pulse rate, and recording of the patient's weight, certain other laboratory investigations may be helpful in determining thyroid activity. In the writer's opinion careful estimations of the basal metabolic rate (BMR) is the more reliable of these other tests. Most hospitals in this country are not equipped for or accessible to PBI or  $^{131}\text{I}$  tracer assays, so that the determination of the BMR may represent the only laboratory aid to diagnosis in such conditions.

### BASAL METABOLISM

*Definition.*—The words basal metabolism are usually used to indicate the energy expenditure of an individual at complete physical and mental rest in a comfortable environment. Basal metabolism may be measured either as the heat given out by the body in a given time or by the oxygen used or carbon dioxide liberated in this time.

#### FACTORS INFLUENCING BASAL METABOLISM

##### (1) SURFACE AREA

The smaller the subject the greater is the surface area in proportion to the mass of the individual. Hence, there will be greater heat loss and the metabolism per unit mass must be greater to maintain the normal body temperature. The metabolism per unit of mass of such animals as rats and mice is very much greater than that of man. It is usually expressed per square metre of body surface either in ml. of oxygen per unit of time or in calories.

taken for 15 to 20 days. A graph can be constructed indicating the metabolic trend before and after taking the thiouracil. If it is lower after treatment with thiouracil it indicates that the patient is suffering from hyperthyroidism.

(iii) *Non-toxic parenchymatous and colloid goitres.* These are liable to occur in districts where the iodine content of the soil is low. This means low iodine percentage in the drinking water and in the food produced on this soil. The basal metabolic rate in such cases is not raised. In some countries minute amounts of iodine are added to the table salt supplied to the public and this, when administered, tends to prevent the development of colloid goitre. A child deprived entirely of iodine from infancy would develop into a dwarfed idiot known as a cretin.

(b) Repeated at intervals of a few days B.M.R. estimations are useful in following the effect of rest in bed, administration of iodine, thiouracil and other medicinal measures on patients suffering from toxic goitre, and in this way aid in assessing the correct time for operative interference.

(c) They are used post-operatively to check the success of the surgical procedure.

(d) A valuable application is in checking the metabolic activity of patients undergoing prolonged medicinal treatment for toxic goitre.

(e) In myxoedema, to determine the degree of hypothyroidism and also to estimate the success of thyroid or thyroxine therapy.

### THE PARATHYROID GLANDS

The chief interest of these glands to the clinical biochemist lies in the fact that they play an important role in the regulation of the calcium (and phosphorus) level of the blood (Fig. 29). In the human subject hypocalcaemia and tetany may occur due to inadvertent removal of the parathyroids when performing subtotal thyroidectomy. Some of the earliest reports of tetany following thyroidectomy came from the surgical clinic of Kocher at Berne. In contrast to the condition just described is hyperparathyroidism or generalized osteitis fibrosa cystica described some years ago by von Recklinghausen. The disease is due to an adenoma or in some cases hyperplasia of a parathyroid gland (or glands). Its chief clinical features are:

- (i) hypotonicity of muscles;
- (ii) pain in bones;
- (iii) hypercalcaemia—accompanied by fall in plasma inorganic phosphorus and a rise in plasma alkaline phosphatase;
- (iv) increased output of calcium by the kidneys with frequent presence of renal calculi;
- (v) irregular decalcification of bones, multicyst formation and spontaneous fracture may often occur.

Estimation of the concentration of the calcium in blood serum is a valuable method of assessing parathyroid activity. This estimation and also the general importance of calcium in health and disease will now be discussed.

*Introduction.*—The principal calcium containing foods are milk, cheese and green vegetables. In certain areas (e.g. South-Eastern Asia) the calcium of the bones of dried small fish makes an important contribution to the calcium of the dietary.

According to Davidson and Anderson two-thirds of the total calcium intake in man is derived in an average diet from milk, and milk contains approximately 0.68 g. of calcium per pint. The recommended requirements for calcium are given in Table XXIII.

It must be remembered that bone undergoes continuous metabolic changes. Deposition of lime salts in and absorption from bone occurs throughout adult life. During the milking period, a cow may lose as much as 20 per cent of the stored body calcium, which is again replaced during the non-lactating period. This calcium comes from the bone, which contains 97 per cent of the total calcium of the body. Vitamin D is an important factor in facilitating the absorption of calcium. Excessive intake of phosphate hinders calcium absorption.

Calcium plays an important part in the following physiological activities:

1. clotting of blood,
2. clotting of milk,
3. excitability and contractility of striated muscle,
4. ossification of bone and formation of teeth,
5. excitability of nerves,
6. permeability of capillary endothelium.

The average calcium content of blood serum is about 10 mg. per 100 ml. It exists in two forms: (a) non diffusible (unable to pass through a collodion membrane) and (b) diffusible (having the power of passing through a collodion membrane). The non-diffusible form is bound to serum proteins, particularly the albumin, and is present to the extent of 4 or 5 mg. per 100 ml. of serum. The diffusible calcium (5 to 6.5 mg. per 100 ml.) is almost entirely ionized, only about 0.25 mg. per cent being non-ionized and in the form of a citrate-like compound. The proportions of serum calcium may be represented as follows:

<i>Mg. calcium per 100 ml. blood serum</i>	
Non-diffusible	4.0 to 5.0
Diffusible	5.0 to 6.5
Ionized	4.75 to 6.25
Un-ionized	0.25

The percentage of calcium in the plasma depends on several factors:

1. The amount absorbed from the intestines.
2. The phosphate content of the plasma. An increase of  $\text{PO}_4$  causes decrease of Ca.
3. The hydrogen ion concentration of the plasma.
4. The secretion of the parathyroid glands.
5. The percentage of plasma proteins. If these are diminished, as in severe albuminuria, the plasma calcium is lowered.

The secretion of parathyroid hormone appears to be regulated by the calcium level of the blood and it effects the mobilization of this element from bone as required. In the event of a phosphate load the hormone also favours, according to Crawford *et al.*, the rapid elimination of this ion by inhibiting its reabsorption by the kidney tubules.

The calcium content of the diet plays an important part in the prevention of industrial lead poisoning. In acute lead poisoning the administration of milk and large doses of calcium lactate aid in fixing the lead in bone, from whence at a later date it can be gradually removed by a diet low in calcium and the administration of ammonium chloride to produce a mild acidosis. Success in the treatment of lead and some other forms of metallic poisoning has been claimed for the intravenous adminis-

tration of Versene (ethylene diamine tetra-acetic acid, EDTA) as its calcium disodium salt.

The blood corpuscles of man are devoid of calcium. Oral administration of calcium in large doses may cause a rise in blood plasma calcium extending over several hours.

### *Estimation of Calcium in Serum*

(Kramer and Tisdall—Clark and Collip)\*

**Principle.**—Calcium is precipitated from the serum as oxalate, and the latter is titrated with potassium permanganate.

**Reagents.**—1. Sodium oxalate solution N/10 used for standardization of  $\text{KMnO}_4$  solution.

2. Ammonium oxalate solution (4 per cent).

3. Potassium permanganate solution N/100 prepared by diluting a N/10  $\text{KMnO}_4$  solution.

4. Ammonia (2 per cent).

5. Sulphuric acid (normal).

**Procedure.**—Into a 15 ml. graduated centrifuge tube of 6 to 7 mm. diameter at the 0.1 ml. mark, introduce 2 ml. of serum, 2 ml. of distilled water and 1 ml. of ammonium oxalate solution (4 per cent) and thoroughly mix the contents by giving the tube a circular movement and tapping the lower end. Allow it to stand for 30 minutes and then mix again. Now centrifuge for five minutes at a speed of 1,500 revolutions per minute; pour off the supernatant fluid and allow the inverted tube to drain for five minutes, and then wipe the mouth of the tube dry with a cloth. The precipitate is stirred by means of a fine stream of 2 per cent ammonia from a wash bottle and 2 per cent ammonia is added up to the 4 ml. mark, taking care to wash down the sides of the centrifuge tube. The contents are again centrifuged and drained as before, and this process is repeated a third time. Two ml. of normal sulphuric acid are now introduced by a pipette and the tube placed in a boiling water bath for one minute. Using a micro-burette, graduated in 0.02 ml., titrate with 0.01N potassium permanganate, till a definite pink colour persists for at least one minute.

**Calculation.**—If  $X$  = the number of ml. permanganate used in the titration and  $Y$  = the amount of 0.01N permanganate re-

\* See also Sulcowicz's test, p. 374.

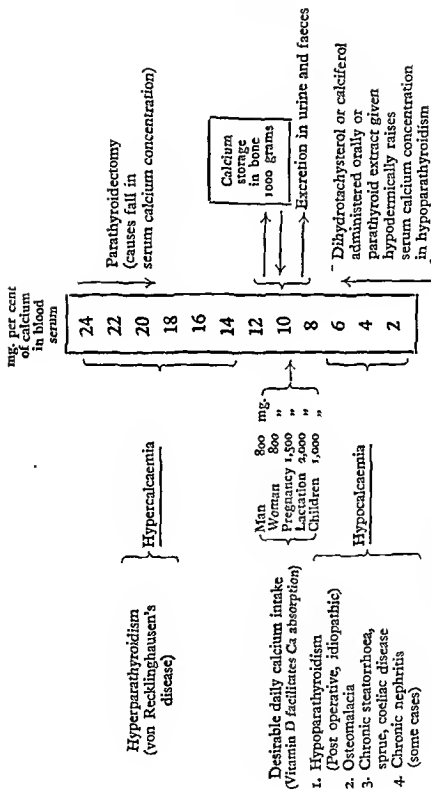


FIG. 29. Some factors influencing the percentage of calcium in the blood serum

quired to impart a pink colour to 2 ml. of fluid, i.e. a blank determination to detect any reducing material in the reagents, then  $(X - Y) \times 10 =$  the number of mg. of calcium per 100 ml. of serum.

*Clinical value of estimation of calcium in serum.*—The post operative level of serum calcium at which tetany develops varies very considerably. Sometimes it may be at 5 mg. per cent, on other occasions, it may be at a higher or lower figure. Repeated blood calcium estimations may be necessary as a guide in treatment of post operative tetany. Such treatment may include the administration of parathyroid extract intramuscularly and the intravenous injection of calcium gluconate. Later, calcium may be given orally and its absorption is facilitated by simultaneous ingestion of vitamin D, in the form of calciferol.

A 0.5 per cent solution of dihydro-tachysterol (ATIO) in sesame oil has also been successfully used by oral administration in the treatment of hypocalcaemia due to parathyroid deficiency. It has remarkable powers of aiding the absorption of calcium.

Von Recklinghausen's disease is usually associated with a blood calcium of 15 to 20 mg. per cent. Not only is blood calcium estimation of value in diagnosing hyperparathyroidism (von Recklinghausen's disease) with hypercalcaemia but such estimations may indicate whether the operation for removal of the hyper-functioning parathyroid tissue has been successful—as judged by a fall in blood calcium.

In the nephrotic syndrome diminution in serum calcium occurs, which is partly accounted for by the dilution of the blood and partly by excretion of calcium bound to protein which is lost in the accompanying proteinuria.

The blood calcium is usually, but not always, low in tetany as is indicated in Table XXIV.

TABLE XXIV  
Blood and Urine Changes in Tetany

Type of Tetany	Blood Chemistry			Ca and P Metabolism
	Ca	P	CO <sub>2</sub>	
1. Parathyroidectomized (post operative)	Low	High	Normal	Urinary Ca excretion very low
2. Parathyroidectomized (idiopathic and traumatic)	Low	High	Normal	Urinary Ca excretion very low
3. Osteomalacia	Low	Low or Normal	—	Neg. Ca and P balance
4. Chronic steatorrhea, sprue, coeliac disease	Low	Low	Normal	Neg. Ca and P balance
5. Infantile (associated with rickets)	Low	Increased	Normal	Loss of Ca and P mostly in faeces
6. At onset of infection (rickets absent)	Normal	Normal	Low if acidosis High if vomiting	No change from normal unless diarrhoea is present
7. Alkalosis (vomiting, NaHCO <sub>3</sub> therapy, hyperventilation)	Normal	Normal	High (Ca and Cl low, P and NPN high if vomiting is persistent)	No definite disturbance in balance unless alkalosis is persistent
8. Chronic nephritis (P retention, renal rickets)	Low	Very high	Low	Urinary Ca and P low

## THE ADRENAL GLANDS

Thomas Addison, in his classical monograph of 1855, first focused attention on the relationship between the adrenal glands and disease and described the syndrome which bears his name. It is usually, but not always, a tubercular disease of the adrenal glands. Cortical deficiency rather than that of the medulla is the cause of most of the symptoms. (Whilst the medulla is of much importance as the source of adrenaline in the body, it will not be further considered in this book.) The disease is characterized by:

- (i) pigmentation of the skin and mucous membranes;
- (ii) low blood pressure;
- (iii) gastro-intestinal disturbances;
- (iv) hypoglycaemia;
- (v) muscular weakness;
- (vi) renal insufficiency with rise in non-protein nitrogen of the blood;
- (vii) dehydration;
- (viii) diminished sodium content of blood serum;
- (ix) increase in potassium content of blood serum.

Bilateral adrenalectomy in an experimental animal is rapidly fatal owing to the profound ensuing disturbances to metabolism, in particular to the imbalance in electrolytes (Na, K). Swingle and Pfiffner in 1929 and 1930 showed that adrenal extracts (free from adrenaline of the adrenal medulla) could be prepared which considerably prolonged the life of the operated animal.

However, it was not until 1935 onwards that any serious attempt was made to isolate in a pure condition the active principle of the adrenal cortex, but due mainly to the investigations of Reichstein in Switzerland, and Kendall and Wintersteiner in the United States, some twenty-eight crystalline steroids have by now been separated; of these seven are active in maintaining life in an adrenalectomized animal. Apart from these lipid-soluble crystalline substances there remained an amorphous fraction highly active in this regard. The latter fraction has recently yielded a new steroid (electrocortin, aldosterone), containing an aldehyde group, which is much more active than the

previously used desoxycorticosterone acetate (DOCA) in maintaining electrolyte equilibrium. It has the further advantage over DOCA, which acts only to favour sodium retention, in that it stimulates excretion of this ion against a sodium load. It seems, therefore, to be the true "salt" hormone of the adrenal cortex.

Of the other crystalline substances cortisone and hydrocortisone (Compound F) are the most potent in their effects on the metabolic pathways of carbohydrate, protein and fat. They are usually referred to as "glucocorticoids" to distinguish their effects from those of the "electrocorticoids" which do not directly influence carbohydrate metabolism. Of the two Compound F appears to be the chief "glucocorticoid" of the human adrenal cortex. Either is able to restore to a normal value the glycogen-depleted liver of the adrenalectomized animal.

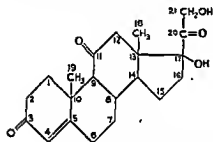


FIG. 30. 17-Hydroxy-11-dehydrocorticosterone. (Cortisone)

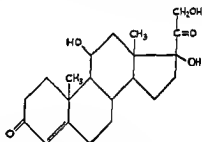


FIG. 31. 17-Hydroxycorticosterone. (Compound F)

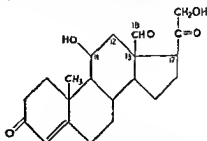
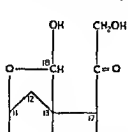


FIG. 32. Aldosterone (a) Ring form. (b) Aldehyde form

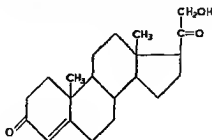


FIG. 33. 11-Desoxycorticosterone (DOCA=21-acetate derivative)

The alteration of the sodium and potassium content of the blood serum is our immediate concern in this chapter as their determination is of importance in the control of treatment of Addison's disease. The reason for the lowered blood serum sodium is the excessive loss of this element in the urine with an associated retention of potassium in the blood.

*Estimation of Potassium in Serum\**

(Kramer and Tisdall)

*Introduction.*—Potassium is found chiefly in the cells of the body, whereas sodium is present mainly in the extra cellular fluid. In blood plasma the approximate potassium content is in the vicinity of 20 mg. per 100 g., but in muscle it is about 320 mg. Some three g. of potassium is excreted in the urine per day, but this is readily replaced by the potassium in the average diet. Many foods are relatively rich in this element, and there is no evidence that anyone suffers from an insufficient potassium intake.

*Principle.*—This method illustrates the formation of an insoluble compound, K cobaltinitrite of the element to be determined. Instead of weighing the small precipitate, this may be estimated titrimetrically by allowing the compound to react with excess permanganate, the nitrate part of the molecule reducing the permanganate. Total permanganate added minus the titrated excess gives a measure of the K cobaltinitrite and hence the potassium.

*Reagents.*—1. Sodium cobaltinitrite reagent (p. 366).

2. N/100 potassium permanganate.

3. 5N sulphuric acid.

4. N/100 sodium oxalate.

5 ml. N/10 sodium oxalate + 1 ml. N/10  $\text{H}_2\text{SO}_4$   
and water to 50 ml. Titrate against N/100  $\text{KMnO}_4$ .

5. Standard potassium solution.

8.914  $\text{K}_2\text{SO}_4$  per litre containing 4 mg. K per ml.  
Dilute 10 ml. to 100 ml.

N.B.—Use serum obtained less than one hour after removal from patient. A dry syringe must be used.

\* Sodium and potassium can be rapidly estimated by the flame photometer but this instrument is not available in all laboratories.

*Procedure.*—To a centrifuge tube add 1 ml. of serum plus 2 ml. sodium cobaltinitrite reagent. Mix and allow to stand one hour. Then add a few ml. of water without mixing and centrifuge. Decant and wash precipitate with water and spin. Repeat until fluid above precipitate is clear, usually two or three washings. Add 5 ml. N/100 potassium permanganate plus 0.5 ml. 25 per cent sulphuric acid and heat in a water bath. If the permanganate is completely decolorized add a further 5 ml. permanganate and heat. Repeat until some of the permanganate remains unchanged (if many additions of potassium permanganate are required the contents of the centrifuge tube will have to be transferred to a beaker). Then add 2 ml. of N/100 sodium oxalate and further 2 ml. if permanganate is not decolorized. Titrate with N/100 potassium permanganate from a microburette until a permanent pink is reached.

*Calculation.*—Total amount N/100 potassium permanganate in ml.—ml. N/100 sodium oxalate  $\times 7.1 =$  mg. potassium per 100 ml. serum. Ml. N/100 potassium permanganate are ml. added to centrifuge tube plus ml. titrated with N/100 potassium permanganate.

E.g. if 5 ml. pot. permanganate are added and 0.5 ml. is used for titration and 2 ml. N/100 oxalate were used, the calculation is  $(5.0 + 0.5 - 2) \times 7.1$ .

$= 24.8$  mg. plasma per 100 ml. serum.

N.B.—The serum must not be haemolyzed.

*Clinical value of estimation of potassium in serum.*—The normal potassium content of blood serum has a range of 18 to 27 mg. per cent. In red blood corpuscles it is about 500 mg. per cent, hence the importance of the use of non-haemolyzed blood for the estimation. After blood is shed potassium passes readily from red cells to plasma (e.g. it may increase by 40 mg. per cent in four hours after blood is shed) so serum must be removed within one hour after collection for the test to be of clinical significance.

In Addison's disease the potassium content of serum is increased and may be 30 to 40 mg. per cent or more.

The demonstration of such an increase in potassium serves as a link in the chain of evidence necessary to diagnose this disease. A diet low in potassium is indicated in the treatment of the disorder.

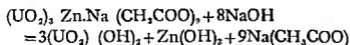
In the rare disease, **Familial Periodic Paralysis**, the serum potassium may be as low as 10 mg. per cent during the attack. The condition is relieved by the administration of potassium salts, e.g. KCl.

### *Estimation of Sodium in Serum*

(Weinbach)

*Introduction.*—The minimal daily requirement of sodium chloride to maintain equilibrium is about two grams, whereas the usual intake varies from ten to fifteen grams. Stoker's and miner's cramp is now recognized as being due to loss of sodium chloride from the body in the sweat, which is not replaced unless, instead of water or other beverages, salted water is taken. A man may lose 3,000 to 4,000 milligrams of sodium in a day by sweating. Three hours' exercise in the sun in summer may result in a loss of sodium chloride in the perspiration equal to a whole day's salt intake. Some of the symptoms of Addison's disease are due to direct loss of sodium from the blood. Excessive vomiting and diarrhoea are indications for increased intake of sodium chloride. The intake should be restricted in congestive cardiac failure and in nephritis with oedema.

*Principle.*—The sodium is precipitated in alcoholic medium as the triple salt, uranyl zinc sodium acetate. Subsequently the salt is titrated with sodium hydroxide, with phenolphthalein as indicator, the uranium and zinc forming amphoteric hydroxides, as follows:



*Reagents.*—1. Uranyl zinc acetate reagent (p. 366).

2. Acetone wash reagent (p. 367).

3. Standard sodium solution (p. 367).

*Procedure.*—With an accurate 0.1 ml. pipette collect 0.1 ml. of serum or whole blood and transfer it to 1.5 ml. of water in a 15 ml. centrifuge tube. From a 1 ml. graduated pipette add, with mixing, exactly 0.4 ml. of 20 per cent trichloroacetic acid. Centrifuge and transfer 1 ml. of the clear supernatant fluid to another 15 ml. centrifuge tube. Add 5 ml. of the uranyl zinc acetate reagent. From a 1 ml. graduated pipette add 0.3 ml. of 95 per

cent alcohol and let stand for 5 minutes. Again add 0.3 ml. of 95 per cent alcohol and let stand for 5 minutes. This procedure is repeated, without greatly disturbing the precipitate, until 2.1 ml. of alcohol has been added, the entire process of precipitation taking about half an hour. Centrifuge, decant, drain on a pad of filter paper, and wipe the mouth of the tube on a cloth. Wash the precipitate once by blowing in 10 ml. of acetone wash reagent, centrifuge, decant, drain on a pad of filter paper, and wipe the mouth of the tube.

The precipitate which is readily soluble in water, is then transferred quantitatively to a 100 ml. conical flask by blowing in three or four 5 ml. portions of water. Add approximately 50 ml. water and 0.5 ml. of 1 per cent phenolphthalein solution and titrate with 0.02N NaOH to a just barely perceptible pink, with a microburette graduated in 0.02 ml.

A blank should be run to determine the amount of 0.02N NaOH which will just give the end point with distilled water.

*Calculation.*—From the equation for the reaction of the uranyl zinc sodium acetate with sodium hydroxide it is seen that  $\text{Na} = 8\text{NaOH}$ . Then the weight of Na in the sample taken would be given by the equation:

$$\begin{aligned} \text{Na in sample} &= (\text{equivalents of NaOH}) (23/8) \times 1000 \\ &= (\text{ml. of 0.02 NaOH required} - \text{ml. of 0.02 N NaOH for blank}) \times (0.00002) \times 23/8 \times 1000 \text{ mg.} \end{aligned}$$

This result  $\times 2000 = \text{mg. Na per 100 ml.}$

The equation reduces to:

$$(\text{actual ml. of 0.02N NaOH}) \times 115 = \text{mg. of sodium for 100 ml.}$$

*Discussion.*—The method described is applicable to the determination of Na in blood, urine, faeces, serum, plasma and cells. Even relatively large amounts of phosphate as found in urines do not interfere with the determinations, although the phosphate precipitates the uranium from the reagent as uranyl phosphate. This is insoluble in water and in the subsequent titration with NaOH there is no reaction with the uranium which has been precipitated as the phosphate.

The end point obtained using phenolphthalein as indicator is rather indefinite. The colour change is very faint and there is no appreciable change in the colour of the solution after the end point, over the range of 0.5 ml.



## XVI

# CHEMICAL AND SPECTROSCOPIC EXAMINATION OF HAEMOGLOBIN AND RELATED PIGMENTS

## INTRODUCTION

The knowledge of some of the simpler chemical tests for haemoglobin, and of the spectroscopic appearance of its solutions and that of related derivatives, is frequently of value in medical work, not only in medico-legal cases involving tests for blood stains but also in the investigation of various clinical conditions.

Pathologically, there may be present in the blood carboxyhaemoglobin, methaemoglobin, methaemalbumin, sulphaemoglobin and nitric oxide haemoglobin, and in the urine oxyhaemoglobin, methaemoglobin, oxymyoglobin, metmyoglobin and free or metallo-porphyrins such as the zinc complexes. Gastric contents may contain oxyhaemoglobin, reduced haemoglobin or acid haematin, and blood may occur abnormally in faeces or in cerebro-spinal fluid.

## HAEMOGLOBIN

Haemoglobin consists of a colourless protein, globin, bound to a prosthetic group, haem. The specific haem of blood, and of many other natural tissue pigments, is an iron complex of protoporphyrin No. 9. The binding of haem to globin appears to involve both the iron and the carboxyl groups of the metallo-porphyrin. The characteristics and important reactions of haemoglobin can best be understood by considering in turn the alterations which can be made to its components (Fig. 34).

### (1) PORPHYRIN UNALTERED, NATIVE GLOBIN

(i) *Ferrous iron*.—When the iron is in the ferrous state, it is able to combine reversibly with such gases as  $O_2$ , CO and NO, without a change of valency. It is not easy to distinguish the

somewhat similar absorption spectra of  $O_2Hb$ ,  $COHb$  and  $NOHb$ .

(ii) *Ferric iron*.—When the iron is oxidized to the ferric state methaemoglobin is formed, which differs spectroscopically from haemoglobin by showing an absorption band in the red and by its inability to combine with the above gases.

## (2) PORPHYRIN UNALTERED, DENATURED GLOBIN

(i) *Ferric iron*.—By heat, certain organic solvents and by strong acids and alkalis, the globin may be denatured. Under most of these conditions the iron-porphyrin prosthetic group remains attached to the denatured protein. The ferric compounds, haematins, do not combine with gases; they show indicator properties as their colour and spectrum differ in acid and alkaline solution. The crystalline salt haematin chloride (haemin) can be readily formed after splitting off the protein with acid. Various reducing agents are used to transform this class of compounds to the ferrous state.

(ii) *Ferrous iron*.—When the iron is reduced haemochromogens (haemochromes) are formed; they have a strong and distinctive spectrum and are able to combine reversibly with carbon monoxide. The denatured protein in this class of compound can be replaced by such bases as pyridine, nicotine, etc.

## (3) COMPLETE REMOVAL OF IRON FROM HAEM

By the action of certain reagents on reduced haemoglobin or  $CO$  haemoglobin, it is possible to split off the iron and protein from the porphyrin. Porphyrins are amphoteric substances, their spectral and other properties are dependent on the acidity or basicity of the environment. Free porphyrins are intensely fluorescent in ultra-violet light, a property of the utmost importance in their detection. They are soluble in most of the common organic solvents. Protoporphyrin No. 9, from haemoglobin, contains four methyl, two vinyl and two propionic acid side chains.\*

\* In older literature the term "haematoporphyrin" usually refers in fact to protoporphyrin. Concentrated sulphuric acid acting on haemoglobin forms haematoporphyrin, in which  $HIOH$  is added to the vinyl side chains ( $-CH=CH_2$ ), making  $-CH(OH)-CH_3$ .

## (4) RUPTURE OF THE PORPHYRIN RING

By the combined action of oxygen and certain reducing agents, e.g.  $H_2S$  or ascorbic acid, it is possible to alter the porphyrin ring and ultimately to form "bile pigment haemoglobins", in which the bile pigment is still attached to both the iron and native globin. In the best known of these, choleglobin, the iron may be oxidized and reduced, and in its ferrous state will combine with CO. Dilute acids split off the iron and protein to form free bile pigments.

Blood haemoglobin yields about 4.5 per cent haem and 0.34 per cent iron. The globin varies a little in different animals; it also varies in the foetal and adult bloods of the same species, and some adults inherit the trait to form abnormal globin. The protein of muscle haemoglobin (myoglobin), confers different characteristics upon this pigment. Although all such haemoglobins possess the same prosthetic group, variations in the globin result in differences of chemical and physical properties which are becoming more widely used to aid clinical diagnosis.

## (A) CHEMICAL TESTS FOR HAEMOGLOBIN

The procedures now described for the detection and specific identification of haemoglobin and some of its derivatives will serve as a preliminary training in technique for the student who later wishes to become more proficient in clinical studies involving the detection of haem and porphyrin pigments. For the chemical tests cloth stained with blood is supplied. In some tests much blood-stained material is needed, whilst in other instances only small quantities are supplied.

*The Benzidine Test*

*Principle.*—Although o-tolidine can be used in the same way, with even greater sensitivity, the original test depends upon the fact that the iron-containing moiety of the haemoglobin molecule acts as a catalyst in the oxidation of benzidine by hydrogen peroxide, and causes the development of a blue compound.

*Reagents.*—1. Glacial acetic acid.

2. Ether (peroxide and hydroquinone free).

3. Alcohol.

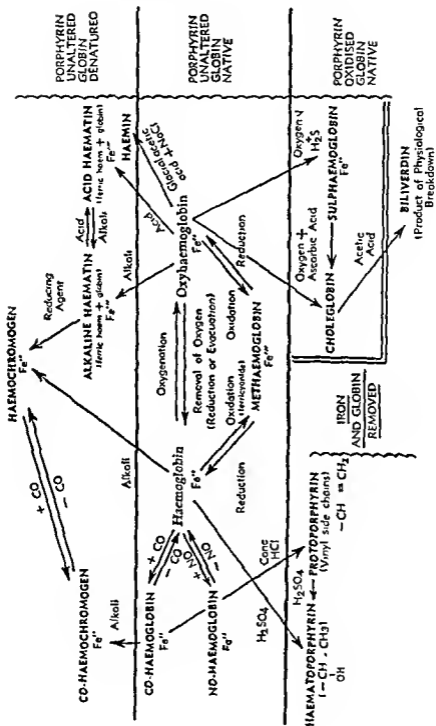


FIG. 34. Haemoglobin and some of its derivatives

*Procedure.*—The blood-stained material is extracted in a watch glass with a little distilled water and the material removed. To the extract is added a few drops of the reagent and two drops of hydrogen peroxide. A deep pink or red colour indicates the presence of blood. A positive reaction may be obtained in as small a concentration as 1 in 5 or 6 millions.

### *Formation of Haemin Crystals*

#### (1) TEICHMANN'S METHOD

*Principle.*—Glacial acetic acid converts haemoglobin into haematin acetate (acid haematin); in the presence of NaCl the more readily crystallizable haemin chloride (haemin) comes out of solution (Fig. 35).

*Reagents.*—1. Glacial acetic acid.

2. Sodium chloride.

*Procedure.*—Place on a microscope slide a drop of watery extract of the material suspected of containing blood and dry thoroughly by holding high over a flame. As an alternative a fragment of the material showing the stain may be used. If the stain is on wood, paper, glass, leather, etc., a few particles should be scraped off with the point of a knife. Add a minute crystal of sodium chloride and a few drops of glacial acetic acid. Cover with a coverslip and heat cautiously over a small flame until bubbles form and the acid partly evaporates. Add more acid and warm again. Repeat this two or three times if necessary, avoiding too great heat or complete evaporation of the acid. Now stand the slide aside for some time in order to allow the crystals to form—half an hour may be necessary for their formation. The crystals, which are dark brown in colour and rhomboidal in outline, lie singly in the field or in clusters, rosettes, etc.

#### (2) NIPPE'S METHOD

*Principle.*—The principle is similar to that of Teichmann's method.

*Reagent.*—Nippé's reagent (p. 360).

*Procedure.*—The technique is similar to that in Teichmann's method, using Nippé's reagent in place of sodium chloride and glacial acetic acid.

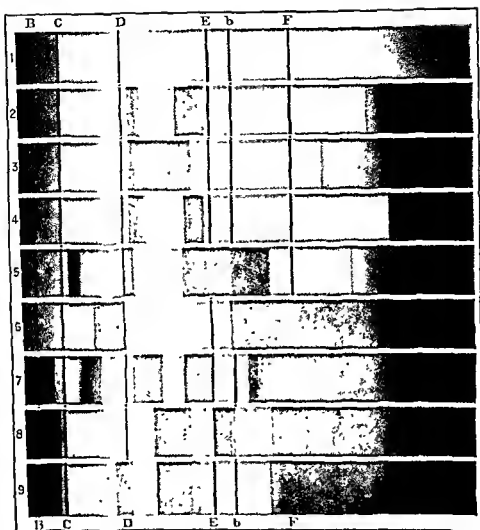


PLATE VI Blood spectra compared with solar spectrum

- 1 Solar spectrum
- 2 Spectrum of dilute solution of oxyhaemoglobin
- 3 Spectrum of reduced haemoglobin
- 4 Spectrum of carbonic oxide haemoglobin
- 5 Spectrum of acid haematin in ethereal solution
- 6 Spectrum of alkaline haematin
- 7 Spectrum of methaemoglobin
- 8 Spectrum of reduced haematin
- 9 Spectrum of acid haematoporphyrin

(From Haliburton, *Handbook of Physiology*)



*Formation of Haemochromogen Crystals*

*Principle.*—The alkali in the Takayama reagent converts haemoglobin into alkali haematin, and the glucose present reduces the alkali haematin, which probably then unites with pyridine to form a haemochromogen.

*Reagent.*—Takayama's reagent (p. 361).

*Procedure.*—Place on a microscope slide a small piece of the material suspected of containing blood. Add two drops of Takayama's solution and cover with a coverslip. The colour changes, through green brown to dark red and then to pink, indicating the formation of haemochromogen crystals which may be recognized under the microscope in the form of salmon pink rhomboids, which, when lying on their sides, give the appearance of single dark lines. The crystals may adhere to form sheaves or resemble fir trees. Crystals usually appear in from one to six minutes, using cold Takayama's reagent, and form more rapidly if the freshly-made reagent is allowed to stand for a few hours before being used. Warming the slide till bubbles just appear greatly accelerates the rate of formation of the crystals. The crystals are a pyridine haemochromogen and show the spectroscopic bands of haemochromogen.

*Clinical value of chemical tests for haemoglobin.*—The benzdine test is extremely delicate and reliable if performed in the manner described. Glaister regards the Kastle-Meyer test as very satisfactory for medico-legal work. Blood stains, even 30 years old, still gave a positive reaction. Great care must be taken that the apparatus is not contaminated with traces of blood from previous examinations. The formation of haemin or haemochromogen crystals is diagnostic of the presence of haemoglobin in the material examined. The formation of haemochromogen crystals using Takayama's reagent is greatly favoured by authorities in forensic medicine.

None of the chemical tests distinguishes human from other forms of blood. Special serological tests must be performed if this distinction is to be made.

One of the most important clinical uses of the tests just described is for the detection of occult blood in the faeces. (See chapter xx. The benzdine test is most frequently used for this purpose. Bleeding from a neoplasm in the alimentary tract may

be detected in this way. Slight persistent haemorrhage from a peptic ulcer may also be revealed when, perhaps, X-ray examination has failed to show the presence of an ulcer. The tests are also of great value in detecting blood in urine, gastric content and in cerebro-spinal fluid as described in chapters xvii, i and xix respectively.

## (B) SPECTROSCOPIC EXAMINATION OF HAEMOGLOBIN AND OTHER HAEM AND PORPHYRIN DERIVATIVES

Certain general directions apply to the spectroscopic examination of all the porphyrin pigments discussed in this chapter.

1. The spectroscope which should be of medium dispersion must be focused by moving the eye-piece till either the Na-emission lines of a Bunsen flame yellowed by contact with a sodium salt, or the absorption bands of the solar spectrum (Fraunhofer lines), are sharply defined. With the simple pocket spectroscope lacking a wave-length scale the Fraunhofer lines are useful in locating absorption bands, but the continuous spectrum of a tungsten lamp is best for determining the relative intensity and width of bands. For rapid and accurate clinical work the Hartridge reversion spectroscope with its ingenious wave-length scale is extremely useful. In recent years spectrophotometers have come into general use for the quantitative estimation of blood pigments (see Plate VI).

The elements in the solar atmosphere to which the Fraunhofer lines correspond are:

<i>Line</i>	<i>Part of Spectrum</i>	<i>Element</i>
B	Red	Oxygen
C	Red	Hydrogen
D	Yellow	Sodium
E	Green	Iron and Calcium
b	Green	Magnesium
F	Blue	Hydrogen
G	Violet	Iron and Calcium

The D, E, b and F lines should be clearly defined in the spectrum of the pocket spectroscope.

2. The red end of the spectrum is kept to the left.

3. Each pigment should be examined in various grades of concentration so that the maximum clearness of the absorption bands may be revealed. Commencing with the strength in which only a small part of the spectrum is seen, water is cautiously added, re-examining after each dilution till the bands finally disappear.

Whole blood is supplied for the student to use in making the various pigments now about to be discussed. It should be diluted as described in the text.

### (1) OXYHAEMOGLOBIN

The oxygenated form of haemoglobin in aqueous solution shows two absorption bands (in yellow and green) between D and E, each band being close to the corresponding Fraunhofer line. The appearances of the bands differ somewhat in various concentrations. Reduced haemoglobin (usually referred to simply as haemoglobin) shows one broad band at the junction of yellow and green. If ammonium hydrogen sulphide or sodium dithionite be added to a solution of oxyhaemoglobin oxygen is removed and haemoglobin will be formed with its characteristic broad band between D and E. The haemoglobin thus formed can be re-oxygenated to form oxyhaemoglobin by simply shaking the solution with air. Reduced haemoglobin may be markedly increased in the blood in some forms of cyanosis. Lundsgaard has shown that about 5 g. of reduced haemoglobin per 100 ml. of capillary blood are necessary to cause cyanosis. This corresponds to an oxygen unsaturation of about 7 vols. per cent. The amount of oxyhaemoglobin in blood has very little effect on the hue of the cyanosis. An anaemic person with less than 30 per cent (5 g. per 100 ml.) of haemoglobin cannot become cyanosed. Haemoglobin is normally formed in the body by reduction of oxyhaemoglobin whilst passing through the capillaries.

### (2) CARBOXYHAEMOGLOBIN

This is the characteristic pigment which is found in the blood of patients suffering from coal gas poisoning, from the inhalation of "fire-damp" in mines, exhaust gases from motor cars and various combustion engines and breech gases from guns. The

two bands of carboxyhaemoglobin occupy a position almost identical with the two bands of oxyhaemoglobin.

It is impossible to detect the former pigment in the presence of the latter by means of a small spectroscope. A reversion spectroscope is necessary for the purpose.

A solution of carboxyhaemoglobin, however, has a cherry-red colour even in extremely dilute solutions, whereas a solution of oxyhaemoglobin of similar concentration has a definite yellow tinge. Another difference is that carboxyhaemoglobin cannot be reduced to haemoglobin by the action of ammonium hydrogen sulphide or sodium dithionite, while oxyhaemoglobin is readily reduced. This is due to the fact that carbon monoxide has an affinity for haemoglobin some 200 or more times that of oxygen. Blood becomes about half saturated with CO in the presence of a mixture of 0.07 per cent of CO in air and marked toxic symptoms are shown in a patient whose blood shows this degree of saturation. Haldane states that as little as 0.02 per cent CO in otherwise normal air produces toxic symptoms if the action be prolonged.

In non-anaemic persons the amount of carboxyhaemoglobin in the blood may be 20 per cent without causing symptoms. However, in those suffering from severe anaemia 20 per cent of COHb in the blood may cause severe symptoms of CO poisoning.

In cases of CO poisoning there is no dyspnoea. This is because the dyspnoeic response to oxygen lack is a reflex initiated in the carotid body when the oxygen tension of the arterial blood is lowered. In CO poisoning the oxygen tension of the blood is normal, despite the fact that its oxygen content is reduced.

Normal blood contains 0.1 to 0.2 volumes per cent of carbon monoxide and this is appreciably increased—doubled or trebled—by smoking.

In cases of death due to carbon monoxide poisoning, the percentage saturation of CO in the blood is usually 80 to 85 per cent, but it may be much less. Since the blood at death is not completely saturated with CO, it must be remembered that, on the addition of sodium hydrosulphite, reduction of the remaining oxyhaemoglobin will occur, and three spectroscopic bands will be present, two sharp bands due to carboxyhaemoglobin and one diffuse band due to haemoglobin.

TABLE XXV

Symptoms Associated with Varying Degrees of Saturation of the Blood with Carbon Monoxide

Per cent Saturation of Blood with CO	Symptoms
0-10	Nil.
10-20	Nil at rest; during exercise tightness across forehead, slight headache.
20-30	Frontal headache may occur at rest; exertion causes dizziness, palpitation and hyperpnoea.
30-40	At rest, headache, frontal or occipital; increased pulse rate, deeper breathing, palpitation, nausea; exertion causes dizziness, dimness of vision, abnormal increase of pulse rate and respiration, sometimes collapse.
40-50	All symptoms more marked; vision, hearing and intelligence begin to be impaired; muscular weakness on attempted exertion, with greater likelihood of collapse. Nausea and vomiting.
50-60	Syncope; increased respiration and pulse rate; coma.
60-70	Coma with depressed heart action and respiration; possibly death.
70-80	Weak pulse and respiration; respiratory failure and death.

(E. M. Killick, *Phys. Rev.* 1940, XX, 313)

From a medico-legal standpoint the presence of nitric oxide haemoglobin must always be considered in cases of suspected carbon monoxide poisoning.

*Preparation of Carboxyhaemoglobin.*—If a dilute solution of blood be shaken with carbon monoxide, or if a stream of coal gas be passed through the solution, carboxyhaemoglobin is formed, and may be recognized as described above.

### (3) NITRIC OXIDE HAEMOGLOBIN

The spectrum of NO haemoglobin is very similar to oxyhaemoglobin, but less sharply defined and is not affected by reducing agents. The NO haemoglobin band in the yellow extends to a slight distance on the red side of the D line. Dilution with water gives a pink colour not as deep as that of carboxyhaemoglobin. Boiling a solution of this pigment gives a pink coagulum, while carboxyhaemoglobin and oxyhaemoglobin give a dull grey coagulum. The pink of the coagulum is due to NO haemochromogen. The red colour of raw salted meat is due to NO haemoglobin, and of cooked salted meat to NO haemo-

chromogen. Nitrate given to an animal causes methaemoglobin to be formed, and also some nitric oxide haemoglobin, the blood being reddish-brown instead of dull brown due to methaemoglobin. Breech gases from guns may contain nitric oxide.

Nitrite-forming organisms are sometimes present in epidemic influenza and in severe broncho-pneumonia, and the cyanosis in these cases may be due to the formation of methaemoglobin and NO haemoglobin. If a coal-miner suffered from such an illness and exhibited cyanosis, carbon monoxide poisoning might be erroneously diagnosed. In poisoning by nitrous fumes some methaemoglobin is formed as well as NO haemoglobin, but the former is reduced post-mortem, leaving NO haemoglobin.

*Preparation of nitric oxide haemoglobin.*—Nitric oxide haemoglobin is formed when nitric oxide is brought in contact with reduced blood, which immediately turns bright scarlet and shows very similar spectroscopic bands to oxyhaemoglobin.

#### (4) ALKALI HAEMATIN

Although of no direct clinical significance, alkali haematin is of indirect importance in that it furnishes an intermediate link between oxyhaemoglobin and haemochromogen.

*Preparation of alkali haematin.*—To a little diluted blood add a few drops of 10 per cent caustic soda. On warming, a brownish colour develops and a faint band is seen with the spectroscope in the yellow (in the region of the D line). This band is usually accentuated by the addition of an equal volume of 96 per cent alcohol.

#### (5) HAEMOCHROMOGEN

In this pigment ferrous haem is combined with denatured globin, and it has a very definite and characteristic spectrum. Of the two bands which are seen in the green when a solution of haemochromogen is examined spectroscopically, that which is midway between D and E is much darker than the absorption band between E and b, and it may persist in solutions so dilute that the latter band has entirely disappeared. This spectrum is of the greatest importance in the detection of blood.

Haemoproteins, found widely distributed in nature, possess very similar absorption bands to the haemochromogens. They play many important roles in the oxidation-reduction changes

in the tissues; cytochrome oxidase and the various types of cytochrome (a, b, c, e and f) are well-known examples.

*Preparation of haemochromogen.*—On the addition of sodium dithionite to alkali haematin, the brownish colour of the alkali haematin changes to the cherry-red of haemochromogen.

The changes may be depicted as:

Oxy Hb + alkali = haematin + globin (denatured).

Globin (denat.) + haematin + reducing agent = haemochromogen.

#### (6) ACID HAEMATIN

Acid haematin may be found in the material ejected in haematemesis. Haematemesis is associated most frequently with gastric or duodenal ulcer, gastric carcinoma and hepatic cirrhosis. Acid haematin is soluble in ether and, if the ethereal extract be examined spectroscopically, four absorption bands may be seen, the most characteristic one being in the left portion of the red. Two fainter bands are seen in the yellow-green (between D and E) and another in the blue (near F). The spectrum is somewhat similar to that of methaemoglobin, but acid haematin may be distinguished by the following features:

- (1) acid reaction,
- (2) solubility in ether,
- (3) conversion into haemochromogen by making alkaline and then reducing with sodium dithionite.

*Preparation of acid haematin.*—To some diluted blood add glacial acetic acid. The solution becomes brownish red and the presence of acid haematin can be shown spectroscopically.

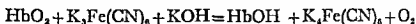
#### (7) METHAEMOGLOBIN

Enterogenous cyanosis is a condition characterized by the presence of methaemoglobin or sulphaemoglobin in the patient's blood. Until recent years, it occurred infrequently, but since the introduction of sulphonamide drugs, it has become more common. Methaemoglobinaemia causes the patient to become a chocolate brown hue, whilst in sulphaemoglobinaemia the skin is a leaden blue colour. There is no accompanying polycythaemia. The pigments are intra-corpuscular and the blood serum is of normal colour. Certain types of reducing bacteria may be responsible for the production of methaemoglobin. Many coal tar

derivatives and other drugs may produce these pigments. Acetanilide, metadinitrobenzene, pyridium and sulphonamide compounds are the most frequent cause of their formation.

Methaemoglobinaemia may be familial owing to an inborn error of the normal cellular reducing mechanism. Blood specimens must be fresh as MHB is rapidly reduced to Hb by the reducing system of the erythrocytes as well as by some drugs.

*Preparation of Methaemoglobin.*—To some diluted blood add a few drops of a freshly-prepared solution of potassium ferricyanide, when the change shown in the following equation occurs:



A brownish colour develops and the solution, when examined spectroscopically, shows bands very similar to those of acid haematin. With the pocket spectroscope the bands appear as one band in the middle of the red and two bands between D and E. It may be distinguished by the following properties:

- (1) neutral or alkaline reaction,
- (2) insolubility in ether,
- (3) reduction to haemoglobin on the addition of ammonium hydrogen sulphide.

#### (8) SULPHAEMOGLOBIN

Sulphaemoglobin is found in the red blood corpuscles and not in the plasma except in cases of severe sepsis in which it may also be present. Its spectrum resembles that of methaemoglobin, particularly in the fact that it has a well-marked band in the red. Acute poisoning by sulphuretted hydrogen does not cause sulphaemoglobinaemia. It is thought that some "intermediary" is necessary to facilitate the combination of  $\text{H}_2\text{S}$  with corpuscular haemoglobin. It has been shown that sulphanilamide catalyzes the reaction between  $\text{H}_2\text{S}$  and haemoglobin to form sulphaemoglobin. The necessary  $\text{H}_2\text{S}$  is probably derived from putrefaction in the bowel. Recent work has indicated that the cause of sulphaemoglobinaemia may be an idiosyncrasy to certain drugs, e.g. sulphanilamide and its derivatives, pyridium or acetanilide, which have been taken by the patient. Cyanosis of varying degrees frequently develops in patients who are receiving sulphanilamide or its derivatives. It has variously been stated as

due to the formation of sulphaemoglobin or methaemoglobin. Actually it may both be due to one or other of these pigments or both.

Sulphaemoglobin does not revert back to haemoglobin like methaemoglobin although its ferrous iron can be oxidized; it remains in the red cells until they are destroyed in the normal way. Although sulphaemoglobin does not combine with oxygen the cyanosis is not a serious toxic manifestation and can usually be disregarded.

In the examination of blood from cyanotic patients preliminary spectroscopic inspection should be made before diluting to about 1 in 10 with water as the band in the red is rather weak; these abnormal pigments are almost always present together with an excess of oxyhaemoglobin. Care should be taken when using sulphur-containing reducing agents to see that sulphaemoglobin is not formed as an artifact during manipulations, particularly in the presence of air; choleglobin can also be formed under similar conditions.

*Preparation of Sulphaemoglobin.*—To some blood diluted with water add ammonium hydrogen sulphide and then hydrogen peroxide. The characteristic pigment is readily formed.

#### (9) METHAEMALBUMIN

Methaemalbumin was first described by N. Hamilton Fairley and Broomfield as occurring in a patient with black water fever. According to Fairley "available evidence suggests that, in intravascular haemolysis of any magnitude, katabolism of some of this circulating haemoglobin results in the production of oxidized haematin (ferric) which combines with serum albumin to form methaemalbumin." The subsequent breakdown of this pigment gives rise to bile pigments, and not porphyrin as earlier assumed.

In the past, methaemoglobin has been universally described in the plasma of diseases associated with intravascular haemolysis and haemoglobinuria. This error has arisen through failure to differentiate spectroscopically between methaemoglobin and methaemalbumin. In black water fever methaemalbumin appears in the blood in addition to haemoglobin and excess of bilirubin. In these cases methaemalbumin is not found in the urine, though

methaemoglobin may be demonstrable in the urine but not in the blood plasma (as was originally thought).

Methaemoglobin and methaemalbumin have a very similar band in the red end of the spectrum, but may be distinguished by:

- (a) Use of the Hartridge reversion spectroscope.
- (b) Schumm's test. This is performed in the following manner: Cover a given volume of serum with a layer of ether and run in with a pipette one-tenth the volume of concentrated ammonium sulphide and mix the solution by shaking. A positive reaction indicating methaemalbumin is shown by the formation of a haemochromogen.
- (c) Dilute ammonium sulphide test. Addition of one drop per ml. of 10 per cent ammonium sulphide disperses the alpha band of methaemoglobin, whilst that of methaemalbumin and sulphaemoglobin persist unaltered.

Methaemoglobin and sulphaemoglobin occur within the corpuscles, methaemalbumin in the plasma. Should there be any lysis of corpuscles containing methaemoglobin or sulphaemo-

**TABLE XXVI**  
**Spectroscopic Examination of Blood for Abnormal Pigments**  
**with Band in Red**

<i>Compound</i>	<i>Red band position (m<math>\mu</math>)</i>	<i>Add Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> reducer</i>	<i>Reduction plus CO</i>	<i>Reduction plus NaOH</i>	<i>Other reagents or initial sample</i>
Methaemoglobin	632	Band disappears. Diffuse Hb band appears	Two bands in green at 570 and 540 m $\mu$ .	Two bands of haemochromogen	Band disappears with cyanide or excess Na <sub>2</sub> CO <sub>3</sub>
Methaemalbumin	613	Band disappears. Two bands appear in green	"	"	Band persists with cyanide or excess Na <sub>2</sub> CO <sub>3</sub>
Sulphaemoglobin	620	No change or slight increase	Band shifts towards yellow at 616 m $\mu$ .	"	"
Choleglobin	630	Band stronger	No change	Band shifts to 619 m $\mu$ .	"

globin the liberated pigments are probably quickly converted to methaemalbumin. Methaemoglobinuria is always secondary and never primary, the methaemoglobin being derived from the oxyhaemoglobin as it passes down the renal tubules.

Fairley suggests that from a biochemical standpoint haemolytic anaemias fall into three groups accordingly as they show: (a) hyperbilirubinaemia alone, (b) hyperbilirubinaemia and methaemalbuminaemia, and (c) hyperbilirubinaemia, methaemalbuminaemia and haemoglobinaemia.

In blood specimens found to show a band in the red end of the spectrum, tests to distinguish between methaemoglobin, methaemalbumin, sulphaemoglobin and choleglobin are best performed as outlined in Table XXVI.

### (C) QUANTITATIVE ESTIMATION OF HAEMOGLOBIN

It seems that in the past the figures accepted for the haemoglobin percentage of normal blood have been too low. Whitby and Britton state that the haemoglobin content of normal blood ranges from 14 to 17 g. with an average of 15.8 g. per cent for a male, and a range of 12 to 15.5 g. with an average of 13.7 g. for a female. Neglecting sex differences the average is 14.5 g. per cent, which is equivalent to 105 per cent on the Haldane scale and 85 per cent by the Sahli method. This is the figure accepted by Price Jones in England, whilst in America it is stated that the percentage is somewhat higher (15.6 g. per cent). The absolute amount is seldom estimated clinically, but rather the relative percentage present in terms of an arbitrarily-fixed normal standard. Clinically, the expression 60 per cent haemoglobin means that the blood contains 60 per cent of the normal, but the makers of different haemoglobinometers use different normal standards and the normal also varies with age and sex. Hence, unless the make of the haemoglobinometer and the age and sex of the patient are known, the interpretation of a percentage of haemoglobin is liable to grave error.

If all haemoglobin estimations were expressed in g. per 100 ml. of blood, confusion would be largely avoided, but this plan has not yet been universally adopted.

There is a physiological diurnal variation in haemoglobin of as much as 10 per cent.

The formation of haemoglobin involves the biosynthesis of the protein globin as well as that of the porphyrin ring, and the incorporation of iron; further factors concerned in the mechanism will be found in the discussion of erythropoiesis. The normal level of iron in blood serum in men is 80-175  $\mu\text{g.}$  per cent and for women 60-160  $\mu\text{g.}$  per cent. Serum iron undergoes similar diurnal fluctuations to haemoglobin; up to 40  $\mu\text{g.}$  per cent in normal cases. After surgical operations of even a mild character serum iron often falls markedly, sometimes down to 40  $\mu\text{g.}$  per cent. Serum can take up ferrous iron to about 400  $\mu\text{g.}$  per cent before toxic symptoms appear, as up to this level the iron is bound firmly to the beta-globulin, transferrin. The serum is normally about 20 per cent saturated but in iron deficiency may be as low as 5 per cent saturated; in haemochromatosis there is full saturation. The serum transport iron is in equilibrium with levels found in the alimentary mucosa and with those in the storage depots of the liver and spleen and also with the iron present in the synthetic phase in the marrow; serum iron is turned over about six times daily.

In idiopathic hypochromic anaemia and in anaemia due to chronic haemorrhage, e.g. from a peptic ulcer, the serum iron is low. It is usually normal in pernicious anaemia, but high values are found in haemolytic anaemia. The iron in the blood corpuscle is present in the haemoglobin and amounts to approximately 50 mg. per cent in the healthy person.

The absorption of iron requires it to be in a soluble and reduced condition. The hydrochloric acid of the gastric juice and the slight acidity of the normal small intestine together with reducing agents in the food, such as ascorbic acids, thus facilitate the absorption of this metal. Saturation of the iron-binding protein, apoferritin, of the mucosa limits further absorption. The ferric iron present in the ferritin so formed is probably mobilized by reduction for transport to the serum where it binds to the transferrin in the ferric state. Quite large amounts of iron go directly to the liver and other organs for storage as ferritin and in normal humans it takes a day or more to be detectable in the red cells and is not maximal until about the tenth day. At the end of the average life span of 120 days of the red cell the iron is stored and then re-utilized; only about 1 mg. is excreted daily. The

usual recommended daily requirement of 10 to 15 mg. iron is probably too high.

The loss of iron in women during menstruation, pregnancy and lactation necessitates an extra quantity of this element in their diet. The average loss of iron at each menstrual period is about 25 to 50 mg., but may be 100 mg. or more. It has been estimated that two-thirds of the iron content of a mixed diet is in a form available for utilization in the body, and approximately one-third of this is absorbed if the body needs iron. Thus in a diet containing 10 mg., about 3.3 mg. of iron may be absorbed daily, and this in a month would replace the iron lost at menstruation. Women of the poorer classes in Aberdeen were shown to consume a diet having on an average 7.5 mg. of iron and some 50 per cent of these were suffering from anaemia of the iron deficiency type.

The following table compiled by Davidson and Fullerton indicates an average demand for iron caused by pregnancy and lactation:

Iron content of foetus at term	400 mg.
Iron content of placenta and uterus	150 mg.
Iron content of blood lost during delivery	175 mg.
Iron content of milk during six months lactation	180 mg.
	<hr/> 905 mg. <hr/>

The amount of iron absorbed from different articles of food varies considerably; thus, most of the iron present in liver is available and 75 per cent of that present in legumes can be absorbed. In meat (muscle), however, only 10 to 25 per cent of the iron is in inorganic form, and therefore available; the balance is present as haemoglobin and haematin compounds, the iron of which cannot be utilized by the human body.

In treating anaemia of the hypochromic type relatively huge doses of iron are found necessary to obtain a satisfactory improvement in the haemoglobin content of the blood. For an increase of 1 per cent of haemoglobin per day the average amount of iron daily ingested as ferrous sulphate was 0.6 g. (containing 180 mg. of iron) or 4 to 8 g. (containing 800 to

1600 mg. of iron) of ferri et ammon. cit., which is a ferric salt. These, it must be remembered, are average figures. Some patients show a satisfactory response to much smaller doses of iron, whilst in others the quantity given must be much greater. The steepening of the diffusion gradient of iron from the intestine to the plasma appears to stimulate synthesis of apoferritin to a limited extent. Nothing is yet known of how the iron becomes incorporated in the porphyrin ring. The content of calcium and phosphate ions in the diet influences iron absorption and unbalanced levels can produce "nutritional haemochromatosis". Achlorhydria reduces absorption when there is a normal dietary, but probably does not greatly affect the absorption when massive doses of iron are given by mouth.

Now that isotope and differential agglutination techniques have established fairly accurately the life of the normal red cell as 120 days the older figures for haemoglobin breakdown need to be reduced by a third. About 10 g. of haemoglobin containing 10 mg. of iron are destroyed daily; this corresponds to an excretion of 330 mg. bilirubin per day, which should appear as stercobilinogen but owing to destruction during passage down the gut only 50 to 200 mg. appears in the stool. If the stool stercobilinogen exceeds about 350 mg. per day then a shortened life span of the red cells is indicated.

The iron split from haemoglobin is re-utilized after storage mainly as ferritin from which it can be readily mobilized; some iron stored as haemosiderin is only slowly made available but it is called upon in times of acute iron shortage such as after haemorrhage. There is no evidence that significant amounts of iron are excreted by the mucous membrane of the large intestine and only a trace is eliminated in the urine. If large quantities of iron are given by mouth only a small fraction is absorbed, most of it passing out by way of the faeces.

We may summarize the factors necessary for normal erythropoiesis as:

(a) *Suitable protein in the diet.*—Experiments by Whipple and others indicate that liver, kidney, egg and muscle are excellent sources of protein necessary for haemoglobin formation. The porphyrin moiety of the haemoglobin molecule is synthesized in the body from such simple compounds as glycine and acetate.

(b) *Vitamin B<sub>12</sub>* (Cyanocobalamin).—The "intrinsic factor" is a mucoprotein which combines with the "extrinsic factor". This combination is necessary for the latter factor, vitamin B<sub>12</sub> to be absorbed by the intestine (see p. 3).

(c) *Iron*.—Discussed in the preceding paragraphs.

(d) *Copper*.—This metal is not a component of the haemoglobin molecule. In animal experiments designed to produce copper deficiency haemoglobin synthesis has been reduced even with adequate iron supply. Copper deficiency has never been proven in humans so the role of copper remains obscure in clinical treatment of disturbed haemopoiesis.

(e) *Vitamin C*.—In scurvy there is usually a moderate degree of anaemia of the microcytic type. Vitamin C is beneficial in these cases in restoring the blood to normal. In pernicious anaemia vitamin C appears necessary to reduce folic acid to folinic acid.

(f) *Thyroxine*.—In myxoedema, anaemia may occur and if due to thyroxine lack may be cured by the administration of thyroid extract. Thyroxine probably acts in such cases in virtue of its being a general metabolic stimulant, including stimulation of the bone-marrow tissue.

(g) *Low oxygen tension in the bone-marrow*.—The action of the various factors previously mentioned as contributing to the formation of red blood corpuscles is facilitated by low oxygen tension in the bone-marrow. The blood of animals exposed to atmospheres deficient in oxygen soon contains more red blood corpuscles than when breathing ordinary air. At high altitudes blood formation is stimulated and a physiological polycythaemia results. It is to be remembered that not all the sinusoids in bone-marrow are open at once. It is in the collapsed anoxaemic sinusoids that erythropoiesis is rapidly occurring. The young cells lie nearest to the lining of the sinusoids and the more mature ones in the centre. The cells in order of their development from the endothelial cells are named megaloblasts, erythroblasts (early and late), normoblasts, reticulocytes and erythrocytes (mature cells).

The scheme in Table XXVII indicates the diseases which arise from certain deficiencies causing failure of maturation of the blood corpuscles.

TABLE XXVII

Diseases arising from certain Deficiencies causing Failure of Maturation of Red Blood Corpuscles

Cell	Deficiency	Resulting disease
Endothelium	?	Aplastic anaemia
↓		
Megaloblast	Intrinsic factor normally formed in stomach and which is necessary for absorption of $B_{12}$ (cyanocobalamin)	Macrocytic anaemia e.g. pernicious anaemia
↓		
Normoblast	(a) Fe (essential component of Hb) (b) Cu, Vitamin C and thyroxine influencing metabolic processes necessary for Hb formation	Microcytic anaemia
↓		
Erythrocyte		

Recent research has indicated that  $B_{12}$  is an essential factor in erythropoiesis. The blood of patients suffering from pernicious anaemia can be restored to normal by the administration of  $B_{12}$  (cyanocobalamin).

#### (1) HALDANE'S HAEMOGLOBINOMETER

This apparatus consists of:

1. A standard tube containing a 1 per cent solution of blood having the percentage of haemoglobin found in the blood of healthy men, and which has been saturated with carbon monoxide. The oxygen capacity of the solution is 18.5 per cent, as determined by the ferri-cyanide method.
2. A 20 c.mm. pipette.
3. A graduated tube in which to dilute the sample of blood.
4. A dropper.

*Method.*—Water rendered faintly alkaline with ammonia is dropped by means of a dropper into the graduated tube up to the mark 20. The lobe of the ear or the end of the finger is pricked

with a needle and the blood is sucked up to the mark 20 in the capillary pipette. It is then gently blown into the graduated tube, where it sinks. The remains of blood in the pipette are washed out with the fluid in the graduated tube. Coal gas is now introduced into the tube by attaching a rubber tube and a suitable pipette to a gas burner and allowing gas to pass for a few seconds. The pipette is withdrawn while the gas is still passing, and the open end of the tube closed with the forefinger. The tube is now inverted several times so as to saturate the haemoglobin with carbon monoxide. Water is now added drop by drop by means of the special dropper supplied. After every few drops the tube is inverted thoroughly to mix the fluid, and frequently compared with the standard. This is continued until a point is reached when the tint of the liquid in the tube corresponds with the colour of the standard. The tube is so graduated that the percentage of haemoglobin can be read off directly as soon as the correct dilution is reached. Water saturated with carbon monoxide may be used to dilute the blood instead of the technique just described. 100 per cent on the Haldane apparatus is equivalent to 13.8 g. of haemoglobin per 100 ml. of blood. When comparing the colours the two tubes should be frequently transposed. The normal for a man is 113 per cent and for a woman 98 per cent.

## (2) SAHLI LEITZ HAEMOMETER

This apparatus consists of a graduated tube into which the blood and hydrochloric acid are introduced and two standard tubes with which the acid haematin solution is compared.

*Procedure.*—Into the graduated tube enough N/10 HCl is introduced to reach the mark 10. With the capillary pipette blood is drawn to the 20 cm. mark, the sides of the pipette are wiped with gauze and the blood blown into the acid. The solution of HCl is sucked up two or three times and expelled until all traces of blood are removed. The tube is then set aside for thirty minutes in order that the maximum degree of colour may develop. HCl solution is now added drop by drop and the contents of the tube well mixed between each addition, by closing the tube with the finger and inverting. This is continued until the colour exactly matches the standard. When this is done the percentage can be read off directly by noting the figure

which the meniscus has reached on the scale. The black scale indicates the percentages, and the red scale indicates how many grams of haemoglobin are contained in 100 ml. of the blood tested. The normal value for this instrument as at present manufactured is 100 per cent (corresponding to 14 g. haemoglobin per cent) for men and 90 per cent for women, but the original 100 per cent of Sahli is equivalent to 17.3 g.

### (3) NEWCOMER HAEMOGLOBINOMETER

In this instrument a standard coloured disc is used, against which the acid haematin formed from the haemoglobin of the blood is matched.

### (4) TALLQUIST'S HAEMOGLOBINOMETER

In this simple method a drop of blood is allowed to fall on a piece of standard absorbent paper and when the humid gloss disappears, it is compared with a paper scale of tints which corresponds to a series of concentrations of haemoglobin, ranging from 10 to 100 per cent. The comparison is effected by moving the blood-stained paper along the scale until the colour is matched. This method, though rapid and of some use in a consulting-room, has a considerable error and should be controlled wherever possible by a more accurate method.

*Note.*—It is wise to have all haemoglobinometers standardized in a biochemical laboratory and checked frequently. Spectrophotometric measurements are beginning to supplant gasometric methods for standardization procedures; they can be made more convenient and accurate than the older methods.

*Colour Index.*—Although the absolute values of red cell and haemoglobin levels are more useful and less misleading, the colour index is still used in many clinical laboratories. The colour index of any sample of blood is obtained by dividing the percentage of haemoglobin by the percentage of the red blood corpuscles, e.g. suppose the red count in a man were 2,500,000 (i.e. 50 per cent normal) and the Hb value 60 per cent, then the colour index would be  $60/50=1.2$ . In macrocytic anaemias (pernicious anaemia) the colour index is above one, whereas in the various types of microcytic anaemias it is less than unity. For detailed discussion of anaemias see Whitby and Britton. *Disorders of the Blood.*

More recent work shows the advantage of such absolute values as: mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, as well as total blood haemoglobin concentration and packed cell volumes.

### MEDICO-LEGAL TESTS FOR BLOOD

Four chief methods of investigation are in use:

- |                 |                   |
|-----------------|-------------------|
| 1. Chemical,    | 3. Spectroscopic, |
| 2. Microscopic, | 4. Biological.    |

#### 1. *Chemical*.—

- (a) The benzidine and other colour tests may be performed, observing the special precautions described when considering these tests.
- (b) Haemin crystals may be prepared by Teichmann's or Nippé's methods.
- (c) Haemochromogen crystals may be prepared by the action of Takayama's reagent. This is probably the best of all the tests.

2. *Microscopic*.—If the blood-stain is fairly fresh, it may be extracted with normal saline and a drop of the solution examined microscopically for blood corpuscles. These may be normal in appearance or more or less crenated.

3. *Spectroscopic*.—A portion of the stain should be extracted with a small quantity of water and examined first for oxyhaemoglobin. If the blood stain be old, the haemoglobin may have been converted into methaemoglobin, which shows its characteristic spectrum. In most cases the best plan is to convert the extract from the blood stain into haemochromogen, as already described, as this compound shows the absorption band between D and E even in extremely dilute solutions.

If the stain is very small, a micro-spectroscope may have to be used.

#### 4. *Biological*.—

- (a) *Precipitin test*.—Having decided by the formation of haemin or haemochromogen crystals or the spectroscopic tests or both that blood is present, the next procedure is to use the precipitin test to distinguish the

species of animal from which it originated. For details of this method a text book of immunology should be consulted.

- (b) *Blood Group tests*.—For further reference to blood groups, see Whitby and Britton, *Disorders of the Blood*.

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## XVII

### BLOOD AND BLOOD PIGMENTS IN URINE

#### HAEMATURIA

The characteristic feature of haematuria is the presence of red blood corpuscles in the urine. The colour of urine which contains blood varies considerably with the amount present. The appearance may be that of blood mixed with water or, on the other hand, the urine may appear only slightly "smoky", or the blood may not even colour the urine.

#### (A) CAUSES OF HAEMATURIA

The causes of haematuria are numerous, the most common being the following:

1. Calculi in the urinary tract.
2. Tumours of the kidney, bladder or prostate.
3. Tuberculosis of the kidney or bladder.
4. Acute nephritis.
5. Injury to the urinary tract.
6. Diseases of the blood: leukaemia, purpura, haemophilia.
7. Diseases of neighbouring organs, but which may involve the urinary tract: carcinoma of the uterus, acute appendicitis or salpingitis.

If the haemorrhage be small and the urine is allowed to stand for some time before examination, the haemoglobin of the red corpuscles may escape, leaving "shadow" corpuscles. In fresh urine of normal concentration the corpuscles present a crenated appearance, whereas in a dilute urine they may appear swollen. If the first urine passed during micturition is blood-stained, and the remainder clear, then the bleeding is probably from the urethra or prostate, whereas if the urine is evenly stained with blood, it indicates renal haemorrhage or a fairly profuse vesical haemorrhage. When the urine is only blood-stained during the

final expulsive efforts of micturition, the lesion is usually in the bladder. A large quantity of blood in the urine usually suggests some form of growth in the bladder or kidney, provided traumatism of the urinary tract can be excluded. If haematuria be associated with the presence of a number of crystals, such as calcium oxalate or uric acid in a urine of acid reaction, the possibility of a renal or vesical calculus is suggested.

Cystoscopy will enable the surgeon to determine whether the bleeding occurs within the bladder or comes from higher in the urinary tract. During the cystoscopic examination blood may be seen issuing from one or both ureteric orifices. In cases where the bleeding is very slight and not obvious to the naked eye, ureteric catheterization will enable samples of the urine to be obtained from each kidney and examined for blood, microscopically. If nephrectomy be contemplated, the efficiency of the kidney which is to remain may be estimated by means of the urea concentration test, or urea clearance test, the urine being obtained by means of the ureteral catheter. If much blood be present, the indigo carmine test is usually precluded, as the excess of blood obscures the field of observation, though by washing out the bladder just prior to injecting the dye, a satisfactory examination may be made.

### (B) DETECTION OF HAEMATURIA

Microscopic examination of the urine is the only satisfactory method of detecting haematuria for, though the urine be obviously blood-stained, this may be due to simple haemoglobinuria. Spectroscopic and chemical examination reveal haemoglobin in urine, but they do not indicate whether red blood corpuscles are present. The centrifuged urinary deposit should be examined with the high power of the microscope for red corpuscles. They must be carefully distinguished from acid sodium urate crystals, which are brown in colour, vary in size and may have numerous spines radiating from their surface, and from yeast cells, which are oval in shape, tend to adhere in short chains with buds attached to the parent cells, and do not give any tests for haemoglobin. The red corpuscles may be normal in shape, swollen or crenated, depending upon the osmotic pressure of the urine.

The microscope will detect blood corpuscles in such small numbers that chemical tests would entirely fail to reveal their presence.

### HAEMOGLOBINURIA

This condition differs from haematuria in that the blood pigment is passed in solution in the urine and not contained in the corpuscles. To distinguish haemoglobinuria from haematuria the fresh urine should be centrifuged and the sediment examined microscopically for red blood corpuscles. On standing a considerable time, disintegration of the red blood corpuscles may occur and a condition which is really haematuria may be interpreted as haemoglobinuria.

When haemoglobin is set free into the circulation by haemolysis, it is removed in three ways:

- by excretion in the urine;

- by absorption into the reticulo-endothelial cells and thence conversion finally to bilirubin and haemosiderin;

- by katabolism in the blood stream to form methaemalbumin

As mentioned in the previous chapter, methaemalbumin was demonstrated in the blood by N. H. Fairley, who states that in incompatible transfusion, black water fever and other intravascular haemolysis in man methaemalbumin and not methaemoglobin is found in the plasma. The absorption spectra of these two pigments is very similar, but it is possible to distinguish them.

#### (A) CAUSES OF HAEMOGLOBINURIA

Haemoglobinuria results from any condition which leads to haemoglobinaemia (free haemoglobin in the blood) caused by laking of red corpuscles within the blood vessels. Some of the factors producing haemoglobinuria are:

##### (1) INGESTION OF CHEMICAL SUBSTANCES

Ingestion of sulphonamides, turpentine, saponin, hexamine, ricin, abrin, may result in haemoglobinuria.

##### (2) INHALATION OF CERTAIN GASES

Arseniuretted hydrogen is notable in this respect. It is taken up by red blood corpuscles and causes their haemolysis, which

may be very rapid and intense. A varying amount of the haemoglobin set free is converted to methaemoglobin (or methaemalbumin) or bile pigment. Most of the liberated blood pigment is excreted in the urine, colouring it a deep red or reddish-brown, depending on the relative amount of methaemoglobin, unaltered haemoglobin, bile pigments or acid haematin present. Excess of bilirubin in the circulation causes the patient to become jaundiced. It is possible that in some cases the precipitation of blood pigments in the renal tubules may be responsible for a considerable degree of impairment of renal function.

### (3) INJECTION OF THE VENOM OF SOME SNAKES

Kellaway, Holden and their associates have studied snake venoms in very considerable detail. One aspect of this work was concerned with haemolysis of rabbit's red blood corpuscles by the venom of the Australian copperhead. The results indicate that this venom and also that of the Australian black snake contains two active agents. One of these haemolysins acts directly on the cells and is most effective at pH 6, whilst the other, which corresponds to the "phosphatidase" of other venoms, acts by the formation of a powerful chemical lysis and is most effective at pH 7. There are always a few red blood corpuscles in the urine after snake bite by Australian snakes. Haemoglobinuria is uncommon in tiger snake bite, but is to be expected after bites by black snake or copperhead. Bites by these latter snakes are rare.

### (4) TRANSFUSION OF BLOOD FROM A DONOR WHOSE BLOOD IS NOT COMPATIBLE WITH THAT OF THE RECIPIENT

Blood transfusion has become of such frequent occurrence in both civil and military medical and surgical practice that it is of the utmost importance to be familiar with the problems of compatibility of blood (see *Disorders of the Blood*, Whitby and Britton).

### (5) PAROXYSMAL HAEMOGLOBINURIA

This condition is due to paroxysmal intravascular haemolysis and is classified by Witts thus:

(i) *Haemoglobinuria from cold (syphilitic paroxysmal haemoglobinuria)*.—This is the commonest type of paroxysmal haemo-

globinuria. Syphilis seems to be a fundamental factor in its causation. Exposure to cold or even washing the hands in cold water may precipitate the condition. It is thought that a lysin in the patient's blood unites with the red cells when the temperature is low, and when the temperature rises haemolysis occurs.

(ii) *March haemoglobinuria*.—This condition occurs in certain individuals after a critical amount of exercise in the vertical position, e.g., marching, running. There is a rise in plasma haemoglobin level above their renal threshold; the amount of lysis is not great. In most cases the renal threshold is about 60 to 80 mg. haemoglobin per cent which is lower than the normal value of 120 mg. per cent, and usually amino-aciduria is also found. Red cell fragility is normal; the pigment is haemoglobin and not myoglobin.

(iii) *Nocturnal Haemoglobinuria*.—These paroxysms occur chiefly but not exclusively at night, or after ammonium chloride feeding. It is found that a lysin in the patient's blood is particularly active at pH 7.0 to 7.2 so that a decrease in blood pH such as occurs during sleep can produce haemolysis. In these patients haemosiderin is generally found in the urine.

(iv) *Myoglobinuria* (Myohaemoglobinuria).—Myoglobin is normally confined to the cytoplasm of the red and cardiac muscle fibres, and its presence in the urine is a sign of muscle damage. The condition may arise spontaneously, in idiopathic cases of paroxysmal paralytic myoglobinuria, in dermatomyositis and in crush syndrome. The myoglobin is rapidly cleared by the kidneys and none can be detected in the plasma, and owing to its ready oxidation it is often found as metmyoglobin in the urine. In acid or old urine specimens it may be present as denatured pigment which can be readily renatured by adjustment to alkaline conditions in the presence of reducing agent.

During the aerial bombardment of England in 1940-1, many persons had limbs pinned under girders and other debris. In such circumstances ischaemia, necrosis and sterile autolysis of striated muscle occurred. In some instances this was rapidly followed by organic renal disease accompanied by myohaemoglobinuria and terminated in uraemia. There was loss of structural integrity of the second convoluted tubules of the kidneys which was apparently induced by the absorption of products

## PORPHYRINURIA AND PORPHYRIA

*Porphyrinuria* is the term used when excessive amounts of porphyrin are found in the urine and the cause is symptomatic, e.g. in lead poisoning, drug intoxication, liver disease, etc. The condition always clears up when the cause of damage is removed. The predominant pigment is coproporphyrin, though traces of other porphyrins can be detected by sensitive chromatographic methods. Coproporphyrin differs from protoporphyrin in the nature of its side chains; it has four methyl and four propionic acid groups. The type of coproporphyrin isomer depends on the relative positions of these eight substituent groups, and of the four isomers possible only coproporphyrin I and coproporphyrin III have been found in nature. In lead poisoning the markedly elevated excretion of coproporphyrin III during the early stages of exposure is a useful indicator of the hazard.

*Porphyria*, although it is accompanied at least intermittently by the excretion of porphyrins or porphyrin precursors in the urine, is the term used to define certain diseases which appear to involve inborn errors of porphyrin metabolism. In congenital porphyria seen most frequently in males the symptoms are marked photo-sensitivity, splenomegaly and haemolytic anaemia of unknown origin. The urine contains extremely large amounts of uroporphyrin I and coproporphyrin I; these pigments are also found in the red cells and serum of patients. Uroporphyrins contain four acetic acid and four propionic acid side chains. The primary cause appears to be centred in the bone marrow which can be shown to give the characteristic red fluorescence of porphyrin under ultra-violet light. The condition is present at birth but may not become serious until later in life when the epidermolysis resulting from sensitivity to light produces gradual obliteration of exposed features.

The other two forms of porphyria are thought to be of hepatic origin, as in such cases the liver contains large amounts of free porphyrin or porphyrin precursor. The most common of all porphyrias is that referred to as "intermittent acute porphyria" in which there appears to be a distinct hereditary pattern. Women in their third decade are the most frequent sufferers. The symptoms are varied ranging from psychosis to abdominal crisis, the one unifying symptom.

is either a dark port wine colour on excretion or on standing rapidly darkens to this colour. The urine contains large amounts of an abnormal substance "porphobilinogen", a mono-pyrrole which condenses to give uroporphyrin and porphobilin on heating under acid conditions. The reddish brown porphobilin gives such porphyria urines their characteristic colour; the uroporphyrin though it may reach 100 mg. per litre usually only contributes a reddish tinge. Most frequently the porphyrin is present as a metal complex, probably zinc, and casual examination often confuses the spectrum of the metalloporphyrin with that of oxyhaemoglobin. However acidification of the urine splits free the porphyrin and allows the characteristic spectrum and intense fluorescence in ultra-violet light to differentiate it readily from haemoglobin. As a confirmatory test for acute porphyria the Watson-Schwartz method for detecting porphobilinogen is ideal, as it is considered to be pathognomic for the condition; it is simple and is also a delicate test for urobilinogen from which it can be easily differentiated. As acute porphyria is definitely familial it is often worthwhile to examine blood relations, so that any "latent" porphyrics can be warned to avoid barbiturate therapy which has been implicated as an agent provoking the condition.

The uroporphyrin excreted usually seems to be a mixture of types I and III, and the coproporphyrin level is raised from normal values of about 120 micrograms per day up to even 15 mg. per day. By more refined chromatographic methods traces of other porphyrins are detectable.

The other form of porphyria also found fairly common is that named "porphyria cutanea tarda", in which skin lesions are prominent and some liver dysfunction, jaundice and colic, is present. No porphobilinogen is detectable but large amounts of free uroporphyrin are found in the urine as well as in the blood and the faeces together with coproporphyrin; the stool also has been found to contain gross amounts of protoporphyrin. The condition usually appears late in life and exacerbates markedly. In both the acute and cutaneous forms of porphyria the liver on acidification fluoresces a vivid red under ultra-violet light but the bone marrow, unlike that in congenital porphyria, does not.

The best method for the detection of porphyrin in urine is

## XVIII

### BLOOD ANALYSIS

Hawk and Bergeim summarize the most important functions of blood as:

- (1) to carry food and water from the intestines and oxygen from the lungs to the tissue cells;
- (2) to carry waste products from the tissue cells to the excretory organs—kidneys, lungs, intestines and skin;
- (3) to carry hormones and in other ways to co-ordinate the activities of various tissues;
- (4) to aid in the defence of the body against disease;
- (5) to aid in the maintenance of hydrogen ion, water, temperature and other equilibria in the tissues.

#### BIOCHEMICAL CHANGES OCCURRING DURING THE STORAGE OF HUMAN BLOOD

The stimulus of military medical and surgical needs has caused rapid developments in the establishment of "blood banks" in various countries. With a fluid of such complex composition it is not surprising to find that it undergoes certain chemical changes on storage. These have been investigated by numerous workers overseas and in this country particularly by Bick. According to this latter observer "the storage of human blood is accompanied by a decrease in the glucose content of the blood, and increase in the reduced glutathione, uric acid, creatinine, non-protein nitrogen and inorganic phosphate. There is little increase in the urea content of the blood." Bick found it possible to keep blood for 14 to 17 days at 0°C before any sign of haemolysis was observed, but during this time the various biochemical changes enumerated above had occurred, the most striking of which was the almost complete disappearance of glucose from the blood. The onset of haemolysis may be delayed by the addition of glucose. The optimum amount of glucose is 0.6 to 0.7 per cent.

## PRESERVATION OF BLOOD FOR ANALYSIS

Blood is prevented from clotting by introducing into the tube in which it is collected, 20 mg. of finely powdered potassium oxalate or two drops of a 20 per cent potassium oxalate solution for each 10 ml. of blood. It may not be convenient to perform blood analysis as soon as the sample has been obtained or the sample may have to be sent from the country to the city for analysis. If there is considerable delay before analysis, glycolysis and other changes may occur. If such a delay is likely a preservative should be added to the blood immediately it is obtained from the patient.

A useful mixture which prevents clotting and glycolysis for two or three days is:

Sodium fluoride	0.1 g.	} per 100 ml. of blood.
Neutral potassium oxalate	0.3 g.	

## INSTRUCTIONS FOR USE OF VISUAL COLORIMETER

- (1) Clean and dry both cups and plungers.
- (2) Adjust, if necessary, the zero readings.
- (3) Fill both cups with the standard solution. Set each at say 20 mm. and adjust the mirror or electric bulb until the illumination of the two half fields is equal.
- (4) Leave the standard fluid in the left-hand cup. Tip back or throw away the solution from the right-hand cup. Wash out this cup with a little of the unknown solution and then fill to a suitable height.
- (5) Make a series of readings (say 6) and average the results. Avoid prolonged staring which causes retinal fatigue.
- (6) The standard and unknown must be approximately the same concentration otherwise considerable error may occur. For this reason two or more standards may be necessary.
- (7) Avoid spilling the contents of the cups on the apparatus.

PHOTORELECTRIC COLORIMETRY (*See Appendix*)

In this book it is unnecessary to describe the full analysis of blood, since the estimation of many of its constituents has no clinical significance. The system of Folin and Wu will be discussed in part in this chapter and the estimates of phosphate and cholesterol in blood will, for convenience, also be included,

This system of blood analysis may be divided into two sections:

- (1) the preparation of a protein-free filtrate;
- (2) The estimation of certain constituents in that filtrate.

### *Preparation of Protein-free Filtrate*

*Principle.*—Tungstic acid is used to precipitate the blood proteins, which it does very completely. Neither creatinine nor uric acid in appreciable amounts is carried down in the precipitate.

*Reagents.*—1. Sodium tungstate solution (10 per cent).

2. Sulphuric acid ( $\frac{1}{3}$ N).

The sulphuric acid, which is accurately standardized, is used in such quantity as to set free the whole of the tungstic acid and to neutralize the carbonate present in commercial tungstates.

*Procedure.*—Transfer a measured amount of blood into a flask, the volume of which is about twenty times that of the blood taken. Dilute the blood with seven times its volume of water and mix well; add one volume of the sodium tungstate and finally one volume of sulphuric acid drop by drop, gently shaking the flask. The mouth of the flask is closed with a rubber stopper and the contents vigorously shaken. The contents change from pink to dark brown in a few minutes, and the coagulum is practically free from air bubbles. It is to be noted that too much oxalate in the blood sample will prevent complete precipitation. In this case, the cautious addition of 10 per cent sulphuric acid drop by drop, with vigorous shaking, may complete the precipitation. The whole of the contents of the flask is now poured on to a filter paper. The filtrate should be as clear as water and give practically no reaction to congo red paper. If it is desired to preserve the filtrate, and the preservative which is described on page 295 has not been used, then toluol may be added in the proportion of two drops for each 10 ml. of blood originally used. Glycolytic fermentation may still occur, but can be prevented by the addition of one drop of formalin. If 10 per cent sulphuric acid has been added for the completion of precipitation, any excess of acid must be neutralized by the addition of sodium carbonate. This is important as a preliminary to the

estimation of blood urea by the urcase method. (By the method of Herbert and Bourne, a filtrate, free from the reducing substance glutathione may be obtained. They use isotonic sodium sulphate solution in the following proportions:— 1 ml. blood, 8 ml. 3 per cent sodium sulphate, 0.5 ml. 10 per cent sodium tungstate and 0.5 ml.  $\frac{3}{4}$ N sulphuric acid. The intact blood corpuscles, containing the glutathione are precipitated by this procedure.)

*Estimation of Non-Protein Nitrogen (Folin and Wu)*

For a full discussion of this method, see *Quantitative Clinical Chemistry*, Peters and van Slyke.

*Estimation of Blood Urea (Folin and Wu)*

For details the reader is referred to *Practical Physiological Chemistry*, Hawk and Bergeim.

*Clinical value of estimation of blood urea.*—This has been discussed in detail in chapter x.

*Estimation of Creatinine in Blood (Folin and Wu)*

*Introduction.*—Creatinine is the anhydride of creatine, the chief non-protein nitrogenous constituent of muscle. The amount of creatinine excreted daily in the urine by a normal person on a meat-free diet is remarkably constant and is independent of the amount of protein in the food or of the total nitrogen in the urine. The creatinine content of the urine is then an index of endogenous nitrogenous metabolism. The creatinine content of normal blood is usually stated to lie between one and two mg. per 100 ml. Doubt has been thrown on these figures by Behre and Benedict, who maintain that creatinine is present only in minute amounts in blood (less than 0.05 mg. per 100 ml.) and that other unknown material is responsible for most of the so-called creatinine reaction. It may be that the chromogenic substance present in blood is closely related to creatinine, being readily converted to this substance in the kidney, and thus accounts for the presence of creatinine in the urine. Whether these views are correct is still a matter of debate. Despite the criticism of Benedict, the present method of estimating creatinine, or its chromogenic equivalent, gives comparative figures which are of a distinct clinical value.

arseno-phosphotungstic acid reagent and sodium cyanide. The blue colour which results is compared in a colorimeter with that given by a standard uric acid solution treated in a similar manner.

The method about to be described does not form part of Folin and Wu's system of blood analysis, but is introduced here, as the method has been found to give more reliable results than that of Folin and Wu.

*Reagents.*—1. Sodium cyanide, 5 per cent solution, containing 2 ml. of concentrated ammonia per litre.

2. Uric acid reagent (p. 363).

3. Uric acid standard solution (p. 363).

*Procedure.*—Into a test tube of about 20 mm. diameter 5 ml. of blood filtrate and 5 ml. of water are introduced. Into another tube are measured 5 ml. of standard uric acid solution and an equal volume of water. To both standard and unknown are added from a burette, 4 ml. of 5 per cent sodium cyanide solution containing 2 ml. of concentrated ammonia per litre, and to the contents of each tube is added 1 ml. of the arseno-phosphotungstic acid reagent. The contents of each tube are now mixed by inversion and at once placed in a water bath at 100°C where the tubes are left for three minutes after the immersion of the last tube, provided that the time elapsing between the immersion of the first and last tube does not exceed one minute. The tubes are now removed to a beaker of cold water for three minutes and then compared in a colorimeter against the standard. If the solution should become cloudy, due to excessive use of oxalate in collecting the blood, repeat the test, adding an extra 5 ml. of water to the standard as well as the unknown, just prior to heating.

*Calculation*—

$$\frac{S}{U} \times \frac{0.02}{1} \times \frac{100}{0.5} = \frac{S}{U} \times 4 \text{ mg. uric acid per 100 ml. of blood.}$$

*Clinical value of estimation of uric acid.*—In gout there may be marked hyperuricaemia. This may be due either to overproduction of urate or under excretion of urate, both being common defects in all human beings and especially marked in those suffering from gout. Man's efficiency in excreting urate is

even lower than that of most common mammals as tubular reabsorption of urate is at least 90 per cent. This apparently purposeless tubular reabsorption causes all human beings to have urates in the body fluids not far below the saturation point. It must be clearly borne in mind that high blood uric acid is not necessarily indicative of gout, since high figures are frequently obtained in leukaemia, lead and mercury poisoning, in toxæmias of pregnancy, in acute nephritis and in chronic nephritis. In uraemia accompanying the terminal stages of azotaemic nephritis, it occasionally amounts to 20 or even 30 mg. per 100 ml. of blood. In gout, the uric acid content of the blood may reach as much as 10 mg. per 100 ml. In uncomplicated gout, uric acid is alone increased, whereas in renal disease, retention of other nitrogenous metabolites, as urea and creatinine, may also occur. Probenecid (Benemid) inhibits the reabsorption of urates and increases the clearance of urate by several hundred per cent and has been of great value in the treatment of chronic tophaceous gout. Some observers believe that familial hyperuricaemia is due to overproduction of uric acid due to an exaggerated "uricotelic vestige".

#### *Estimation of Chlorides in Blood or Plasma (Whitehorn)*

*Introduction.*—The chloride of the blood is completely dialyzable, none of it being in the colloidal state. The chloride content of whole blood (expressed as NaCl) varies from 0.45 to 0.53 per cent, and of plasma from 0.56 to 0.62 per cent. There is thus a higher concentration of chlorides in the plasma than in the corpuscles, hence it is advisable in clinical work to determine the chloride content of the plasma rather than the whole blood, since in the latter a fall in the number of red cells, such as occurs in anaemia, would lead to a rise in the chloride content. Under physiological conditions only slight variation in the plasma chloride occurs. Excessive ingestion of sodium chloride leads to a retention of water and the maintenance of the normal chloride level. Chloride is a "threshold" substance and it is only when its concentration in the plasma exceeds 0.56 per cent that it is excreted in appreciable quantities.

*Principle.*—To the protein-free filtrate from plasma is added a known amount of silver nitrate and a little nitric acid, which

- Reagents.*—1. Trichloroacetic acid 10 per cent.  
 2. Ammonium molybdate (No. 1) (p. 362).  
 3. Ammonium molybdate (No. 2) (p. 362).  
 4. Aminonaphtholsulphonic acid 0.25 per cent.  
 5. Standard phosphate solution (p. 362).

*Procedure.*—Introduce 8 ml. of 10 per cent trichloroacetic acid into an Erlenmeyer flask. Rotate the flask gently and run in 2 ml. of plasma. Close the flask with a rubber stopper, shake vigorously and then filter through an ashless filter paper. Measure 5 ml. of the filtrate into a test tube marked at 10 ml. Add 1 ml. of 2.5 per cent ammonium molybdate in 3N sulphuric acid. Mix well. To 5 ml. of the standard phosphate solution in a similar tube, add 1 ml. of 2.5 per cent ammonium molybdate in 5N sulphuric acid and mix. Add 0.4 ml. of the sulphonic reagent and water to the mark 10 ml. in both the standard and unknown. The reading in the colorimeter is made in about 5 minutes and repeated a little later if the colour is particularly strong.

*Calculation.*—

$$\frac{S}{U} \times \frac{0.04}{1} \times \frac{100}{1} = \frac{S}{U} \times \frac{4}{1}$$

= mg. phosphorus per 100 ml. of blood.

*Clinical value of phosphate estimation.*—In rickets of children the inorganic phosphorus may fall from a normal of 5 mg. to 2 mg. per 100 ml. of plasma, or even lower. Howland and Marriott state that if the serum calcium and serum inorganic phosphorus values be expressed in milligrams per 100 ml. of serum, then the product of these figures is between 50 and 60 in the normal child. If it should be below 30, rickets is invariably present. In countries of the Northern Hemisphere rickets has its maximum incidence in March and, according to Hess and Lundagen, the phosphate of the plasma is increased in summer and falls in winter. It may be that the seasonal variation in ultra-violet solar rays is responsible for these facts, as it is known that exposure to artificial ultra-violet radiation, which induces the formation of the anti-rachitic vitamin from ergosterol, increases the phosphorus content of the blood and improves the calcification of bone. In severe nephritis, the plasma phosphate may be markedly increased, so that figures of 20 to 30 mg. may

be found. In such circumstances the increase of phosphate content of the plasma is accompanied by a retention of non-protein nitrogen. Acidosis may be a sequel of marked phosphate retention. Following major fractures in adults, there is usually a rise of the inorganic phosphorus of the blood extending over a period of three or four weeks.

*Estimation of Cholesterol in Blood (Day and Bolliger)*

*Introduction.*—Cholesterol ( $C_{27}H_{48}O$ ) is a monohydric, unsaturated, secondary alcohol. It is one of the most important members of the group of animal sterols. These sterols contain a ring known as the phenanthrenecyclopentane or "cholane" ring. It is one of the most massive ring systems with which living cells have to deal and is very stable. Hydroxyl groups or double bonds in the ring serve as centres for chemical change. Slight modification of the molecule at such sites may profoundly alter its physiological properties, but the ring itself remains intact, resisting any deep-seated structural alteration. The structural difference between ergosterol and vitamin D is very slight, and yet the physiological differences are remarkable. Research in recent years has shown the great importance of sterols in physiological processes. They are constituents of all cell membranes and are found particularly in the ovaries, testes, nervous system, adrenal cortex and red blood corpuscles.

The cholane ring is found in the members of the following series, each of which is of considerable clinical interest:

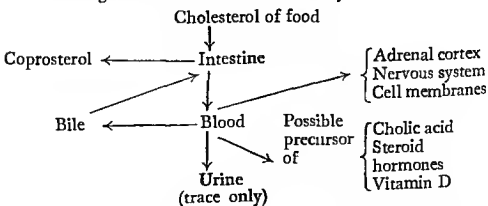
- (1) cholesterol and its reduced form coprosterol;
- (2) the bile acids;
- (3) ergosterol and its irradiated product vitamin D;
- (4) androgenic hormones (testosterone, androsterone);
- (5) oestrogenic hormones (oestradiol, oestrone);
- (6) corpus luteum hormone (progesterone);
- (7) adrenal cortical hormones.

The pathologist has now a particular interest in the sterols as it has been shown that some sterols are carcinogenic.

Plants can readily synthesize some sterols. Even a simple mould such as *Mucor mucedo* can form a sterol when grown on a medium containing only lactose as a source of carbon. The

common plant sterols such as sitosterol and stigmasterol are not absorbed from the human alimentary canal. With the exception of ergosterol no plant sterol is found in the animal body. The cholesterol content of the blood is largely determined by the diet, but there is little doubt that some cholesterol can be synthesized in the human body as well as being formed during the disintegration of old red blood corpuscles. In the diet it is derived from the cholesterol in animal foods such as eggs, brains, butter, cream, liver and kidneys. It is excreted almost entirely in the bile and is in part reabsorbed into the blood from the intestine and in part converted into coprosterol and excreted in the faeces. A trace only appears in the urine normally, but, in the nephrotic syndrome with marked proteinuria, considerable amounts of cholesterol may be excreted by the kidneys. It is present as free cholesterol in the blood corpuscles and in this form and as esters in the plasma. The normal variation of blood cholesterol is 0.10 to 0.20 per cent during fasting. McEachern and Gilmour showed that administration of 2 to 10 g. of cholesterol in a mixture of alcohol and skimmed milk has very little effect on the height of the blood cholesterol. Ordinary meals cause a barely detectable rise in the cholesterol content of the blood. Cholesterol is carried in the blood chiefly adsorbed to plasma globulin.

#### Diagram to illustrate Cholesterol Cycle in Man



*Principle.*—The blood is dried on filter paper and extracted with chloroform. The extract is treated with acetic anhydride and sulphuric acid, and the colour formed estimated in a colorimeter.

*Reagents.*—1. Carbon tetrachloride.

2. Chloroform.

3. Acetic anhydride.

4. Sulphuric acid (pure).

5. Standard cholesterol solution in chloroform (0.1 per cent).

*Procedure.*—1 ml. of the patient's blood is dropped on to two filter papers (seven cm. No. 40 Whatman). These are placed on watch glasses in an incubator at 60°C till dry (about one hour). The two papers are then placed together, folded in half and rolled up and introduced into an ordinary test tube and chloroform added to little more than cover them. The tube is then stood in a small beaker containing carbon tetrachloride and this, in its turn, is placed in a larger beaker containing water. This latter beaker is then heated. As long as the chloroform boils but the carbon tetrachloride does not, the temperature for extraction is correct. If the  $\text{CCl}_4$  begins to boil, remove the flame. Extraction should be continued for half an hour and then the extract transferred to a standard flask. Fresh chloroform is added to the filter papers and extraction again performed for a quarter of an hour and the extract added to the standard flask. This extract is now made up to a known volume, e.g. 15 ml., with chloroform and an aliquot of the extract, e.g. 5 ml., taken for colorimetric estimation. To it is added 2 ml. acetic anhydride and 0.1 ml. conc. sulphuric acid. The standard is simply prepared by taking 1 ml. of a stock solution containing 0.1 per cent cholesterol, which is made up to 15 ml. with chloroform, and to 5 ml. of this is added 2 ml. acetic anhydride and 0.1 ml. conc. sulphuric acid. In each case the mixtures are allowed to stand for 10 minutes in the dark and then they are compared in a colorimeter.

*Calculation.*—

$$\frac{S}{U} \times \frac{1}{1} \times \frac{100}{1} = \text{mg. cholesterol per 100 ml. of blood.}$$

Some advantages of this method are:

(1) no elaborate apparatus is required;

(2) the extraction time is only three quarters of an hour;

- (3) the regulation of temperature in drying and extraction prevents the formation and extraction of decomposition products of haemoglobin, which would give a yellow-green colour in the unknown, while the standard is blue-green.

*Clinical value of cholesterol estimation.*—In diabetes mellitus the serum cholesterol may be 300 mg., or more per 100 ml. of blood. In gross lipaemia, over 3,000 mg. per cent has been recorded. In the nephrotic syndrome, high cholesterol findings are frequent, and are associated with oedema, albuminuria, low protein content of the plasma, the globulin frequently being in excess of the albumin, and practically a normal nitrogenous excretion. Hypercholesterolaemia may also occur in arteriosclerosis, and in the later months of pregnancy. According to Slemons and Curtis, the normal placental membrane is permeable for free cholesterol, but impermeable for cholesterol esters, hence cholesterol esters are frequently absent from foetal blood. Opinions differ as to the concentration of cholesterol in the blood in cholelithiasis. Chauffard, representing the French school, subscribes to the view that hypercholesterolaemia is an important factor in the development of gall stones. On the other hand, many American investigators deny that in cholelithiasis such a rise in blood cholesterol takes place. Post operative myxo-oedema is accompanied by hypercholesterolaemia. In pernicious anaemia, in secondary anaemias, and in toxic goitre diminished cholesterol content of the blood is the rule.

*Estimations of Blood Serum Proteins by Copper Sulphate  
Method of Measuring Specific Gravity of Serum*

(Modified technique of Phillips, van Slyke, Dole, Emerson,  
Hamilton, Archibald)

*Introduction.*—Protein is of the greatest importance in human nutrition. Of the 22 amino acids of special biochemical interest at least 8 and probably 10 are essential for normal human metabolism. The protein in the diet is the ultimate source of that found in the blood plasma. During the process of digestion the protein molecules are resolved into their component amino acids which after absorption are utilized by the liver to form the proteins of the plasma. The chief of these are albumin, globulin

and fibrinogen in the proportion of approximately 5 g., 2g., and 0.2 g. in each 100 ml. of plasma. Not only are albumin and globulin found in the plasma, but they are present in tissue stores and can be called upon in times of need. Elman *et al.* calculated that a reduction of 1 g. in the total circulating protein, e.g. in starvation, indicates a loss of 30 g. of body protein. Thus a man with a plasma volume of 3,500 ml. and with 4 g. of albumin per cent would have a total plasma albumin of 140 g. A reduction of this protein concentration from 4 to 3 g. per 100 ml. would mean a reduction in the total plasma albumin from 140 to 105 g. or a loss of 35 g. Such a loss of plasma albumin would represent a loss from the body of approximately 1,050 g. of body proteins. Albumin is regenerated at the rate of 25 g. or more per day.

Two of the chief functions of the plasma are firstly in connection with maintaining the normal osmotic pressure of the blood and secondly in determining the immunity reactions of the organism. Serum albumin has a molecular weight of 70,000 compared with globulin of 150,000. It also has a concentration in the plasma more than double that of globulin. Hence it has a much greater osmotic effect than the latter. Oedema is likely to develop if the plasma protein (particularly albumin) loss is excessive as in the nephrotic syndrome or if its formation is sub-normal as in time of famine.

The importance of proteins in resistance to infection has been especially studied by Cannon *et al.* These authors suggest that a protein matrix exists within the tissues from which anti-bodies arise. This matrix is composed of globulin from which is derived the anti-body gamma globulin of the immune serum. Globulin synthesis is fundamental to the problem of anti-body formation yet more attention is usually given to fall in albumin content of the blood plasma. The ordinary chemical method of fractionation does not demonstrate the gamma globulin as an entity and yet it is this gamma globulin which is the anti-body. In the nephrotic syndrome the gamma globulin may be reduced, whilst the alpha and beta fractions of globulin are increased. Gamma globulin contains lysine, methionine, tryptophane, threonine and leucine, and it is obvious that this protein can be synthesized only if the diet contains protein having the above mentioned amino acids in their composition. Cannon *et al.* have

shown that experimental animals subjected to prolonged protein under-nutrition exhibit a pronounced loss of capacity to produce anti-bodies and to resist infection.

In the original report by the authors mentioned above, the copper sulphate method for measuring specific gravities of whole blood and plasma is described with line charts for calculating plasma proteins, haemoglobin and haematocrit from plasma and whole blood gravity.

In the present description their method is adapted to the estimation of blood serum proteins by the copper sulphate method of measuring specific gravity of serum and then by reference to a table the percentage of serum protein. It is thought desirable to estimate the haemoglobin by one of the methods described in chapter xvi.

*Principle.*—This consists of letting drops of blood serum fall into a graded series of solutions of copper sulphate of known specific gravity and noting whether the drops rise or fall in the solutions. Using Table XXIX (p. 316) the percentage of serum protein corresponding to a serum specific gravity can be read.

*Reagents and Apparatus.*—

1. Glass syringes, 5 or 10 ml. capacity.
2. No. 20 hypodermic needles.
3. Rubber tourniquet.
4. Centrifuge. (This can be omitted if one can wait for the blood to clot and a few drops of serum to separate).
5. Medicine droppers.
6. Standard copper sulphate solutions. (For method of preparation, see p. 369).

*Procedure.*—The drop of serum is delivered from a height of about one cm. above the solution from a medicine dropper. It is preferable to use small drops for the reason that they permit more tests before the standard solution must be changed. Therefore a medicine dropper with a fine tip is preferable to a coarse one. When the drop is delivered it is convenient to steady the dropper on the edge of the bottle.

The delivered drop breaks the surface film of the solution and penetrates 2 to 3 cm. below the surface; within 5 seconds the momentum of the fall is lost (it is encased in a sac of copper-

proteinate) and the drop then either begins to rise or becomes stationary or continues to fall. The gravity of the drop relative to the solution does not change appreciably until the drop has been immersed in the solution for another 10 or 15 seconds, and there is ample time to note its behaviour during this interval. If the drop is lighter than the test solution it will rise, perhaps only a few millimetres, and may begin to sink immediately afterwards. If the drop is of the same gravity as the standard test solution, it will become stationary for this interval, and then fall. If the drop is heavier it will continue to fall during the interval. *In summary, the behaviour during the 10 seconds after the drop has lost the momentum of its fall into the solution indicates whether the drop is lighter or heavier than the test solution; if it rises at all during this period it is lighter than the standard.*

N.B.—The size of the drops does not have to be constant. Hence no special pipette is needed for delivering the drops. No temperature correction is needed, because the temperature coefficients of expansion of the copper sulphate solutions approximate those of blood and plasma. This method is capable of measuring the specific gravity to  $\pm 0.00005$ , which is more than 10 times the accuracy required. The copper sulphate solution automatically cleans itself after each test, because within a minute or two after the test is completed the material of the drop settles to the bottom as a precipitate. The standard  $\text{CuSO}_4$  solutions are prepared by dilution of a stock solution which has 1.1000 times the density of water at  $25^\circ\text{C}$ .

For precise work, viz. gravities accurate within  $\pm 0.0002$ , a "laboratory set" of 60 copper sulphate solutions graded at intervals of 0.001 in specific gravity are used; 20 solutions cover the plasma or serum range 1.016–1.035.

The following example shows how, by bracketing on the probable extremes of a serum's gravity range and then testing intermediate points, one can find the correct gravity with not more than 4 drops to within  $\pm 0.0002$ . The serum was expected to be of normal or greater concentration. Four successive drops gave the following results, in which the figures indicate the gravities of the standards, and + or – indicates that the serum was heavier or lighter than the standard: 1.027, +; 1.031, –;

1.029, +; 1.030, -. The serum was heavier than 1.029 and lighter than 1.030 and could therefore be placed at 1.0295 with an error less than  $\pm 0.0004$ . By noting the relative rate of fall or rise in the two adjacent solutions, 1.029 and 1.030, it was further obvious that the serum was nearer 1.029 than 1.030. Being less than 1.295 and greater than 1.0290 it could be placed at 1.0292 or 1.0293 with an error not greater than  $\pm 0.0002$ .

*Calculation.*—A table for the conversion of serum gravities to serum proteins concentration is shown on p. 315. Plasma proteins per cent are higher by the 0.2 g. of fibrinogen per 100 ml. present in the plasma.

### SPECIAL POINTS AND PRECAUTIONS

(i) *Surface film effects in the analyses.*—Occasionally a drop will fail to make a clean break through the surface film of copper solution, and remain attached by a tentacle to the film. In this case the drop is detached from the film by tapping the tube, and a fresh drop is tried. After each test, one makes sure that the surface film is left clean and free from fragments. If any are left on the film they are likely to prevent a clean break-through of the drop in the next test. Fragments caught in the surface film can usually be detached by tapping the tube; they then sink to the bottom. Sometimes, however, a fragment of fatty nature or holding a bubble will continue to float on the surface. Such fragments are removed with a wooden applicator stick.

(ii) *Avoidance of bubbles.*—Even a minute air bubble attached to a drop of serum will make it float. While drawing into a dropper or capillary make sure that the tip is kept immersed. When a drop is expelled into copper sulphate solution enough excess serum should be present to leave some in the dropper.

(iii) *Temperature effects.*—The method requires no special attention to temperature.

(iv) *Number of analyses that can be made before renewal of the copper sulphate is necessary.* Tests have shown that a standard solution will receive about one-fortieth its volume of serum or one *small* drop per ml. under the conditions of the tests, before the gravity of the standard is changed by 0.0005. The change is to decrease the gravity. A 4-ounce bottle of standard serves for about 100 tests.

TABLE XXIX

Table indicating grams per cent of Serum Proteins  
corresponding to Serum Specific Gravity

<i>Serum specific gravity</i>	<i>Serum proteins (g./100 ml.)</i>	<i>Serum specific gravity</i>	<i>Serum proteins (g./100 ml.)</i>
1.03482	10.00	1.02482	6.4
1.03456	9.9	1.02454	6.3
1.03428	9.8	1.02426	6.2
1.03402	9.7	1.02398	6.1
1.03374	9.6	1.02370	6.0
1.03348	9.5	1.02342	5.9
1.03322	9.4	1.02314	5.8
1.03294	9.3	1.02286	5.7
1.03266	9.2	1.02258	5.6
1.03238	9.1	1.02230	5.5
1.03210	9.0	1.02202	5.4
1.03182	8.9	1.02174	5.3
1.03152	8.8	1.02146	5.2
1.03124	8.7	1.02118	5.1
1.03096	8.6	1.02090	5.0
1.03068	8.5	1.02062	4.9
1.03040	8.4	1.02034	4.8
1.03010	8.3	1.02006	4.7
1.02982	8.2	1.01978	4.6
1.02954	8.1	1.01950	4.5
1.02926	8.0	1.01922	4.4
1.02898	7.9	1.01894	4.3
1.02872	7.8	1.01866	4.2
1.02844	7.7	1.01838	4.1
1.02816	7.6	1.01810	4.0
1.02788	7.5	1.01782	3.9
1.02760	7.4	1.01754	3.8
1.02732	7.3	1.01726	3.7
1.02706	7.2	1.01698	3.6
1.02678	7.1	1.01670	3.5
1.02650	7.0	1.01642	3.4
1.02622	6.9	1.01614	3.3
1.02594	6.8	1.01586	3.2
1.02566	6.7	1.01558	3.1
1.02538	6.6	1.01530	3.0
1.02510	6.5		

NOTE.—Owing to the absence of fibrinogen from blood serum, plasma proteins are higher by 0.2 g. per cent than serum protein.

*Weichselbaum Method for Estimation of Serum Protein  
(Biuret Method)*

(1) TOTAL PROTEIN ESTIMATION

Reagent.—Biuret reagent (Weichselbaum), (p. 367).

*Procedure.*

(1) Dilute serum 1/20 with normal saline, i.e. 0.50 ml. of serum—9.50 ml. of saline.

(2) Pipette 2 ml. of diluted serum into a hard glass test tube. Add 8 ml. of Biuret reagent (dilute Weichselbaum). Mix well.

(3) Place tube in a beaker of water at 32° — 33°C for 30 minutes. If the whole is incubated at 37.5°C the temperature of the contents of the tube is maintained at 30°C.

(4) Read in a photo-electric colorimeter, using a water blank adjustment to zero before reading (624 Ilford filter). Read percentage of protein from graph.

## (2) ESTIMATION OF THE A/G RATIO OF SERUM PROTEIN

*Reagents.*—1. Sodium sulphate (22.6 per cent).

2. Ether.

3. Biuret reagent and its standardization and also colorimetric standardization (pp. 367-8).

*Procedure*

(1) Measure 0.5 ml. of serum into a centrifuge tube.

(2) Add 9.5 ml. of 22.6 per cent sodium sulphate. Stopper and mix by inversion, not shaking. Pipette 2 ml. of the mixture *immediately* into a hard glass test tube for the *total protein estimation*.

(3) Place the centrifuge tube containing the rest of the mixture in the incubator for 10 minutes.

(4) Add 3 ml. of ether. Mix, cap, and centrifuge hard.

(5) Transfer 2 ml. of the clear aqueous layer to a hard glass test tube for the *albumin estimation*.

(6) Add 8 ml. Biuret reagent to both tubes. Incubate as in (1) 3 above.

(7) Read in the photo-electric colorimeter against a water blank.

(8) Read percentage protein off the same graph as used for the total protein estimation.

N.B.—The 22.6 per cent sodium sulphate solution is kept in the incubator at 37°C.

The centrifuge tube and any pipettes used in the estimation are placed in the incubator for one half hour before using. These precautions are necessary to prevent crystallization of the sodium sulphate especially in cold weather.

## CLINICAL VALUE OF ESTIMATION OF SERUM PROTEINS

The author is indebted to Phillips *et al* for many of the comments in this section.

Estimation of total serum proteins has the obvious disadvantage that no information is obtained concerning the concentration of the individual proteins, nevertheless it may be of much value to country practitioners who have no special laboratory facilities. For details of the separate determination of these proteins the reader is referred to larger text books on biochemistry.

So far as the interpretation of serum protein determinations is concerned, one may say that an abnormal value is definite proof that one of the factors controlling concentration has been disturbed. However, a normal protein concentration is not final proof that the factors controlling the concentration are all working normally, for there may be abnormalities with opposite effects which balance; in such case the serum gravity fails to indicate the pathological conditions that nevertheless exist. Such a balance may occur in either an acute condition, such as haemorrhage, or a chronic one, such as liver cirrhosis.

Haemorrhage, even of great severity, does not affect blood concentration at once; if dehydration is present, and no fluids are taken, haemodilution may not be observed for as long a period as 12 hours. Usually, however, interstitial fluid begins to enter the circulation soon, and in a few hours the resultant fall in concentrations of both serum proteins and haemoglobin may be marked.

Liver cirrhosis decreases the albumin, but may increase globulin formation, with a resultant normal or even high total protein concentration in the plasma.

Such opposing abnormalities may not be frequent. Their existence, however, shows that a normal serum concentration is not certain proof of a normal plasma volume, nor of a normal condition of the metabolic factors that control albumin and globulin formation. Serum gravity measurements can be interpreted only in conjunction with the history and clinical findings. (Phillips, *et al*.)

*An estimate of the percentage of serum proteins is of importance in directing the treatment of the following disorders:*

## (1) MEDICAL CONDITIONS

(i) *Diseases of the kidneys*

Type II nephritis (nephrotic syndrome). Gross albuminuria with lowering of serum protein level may occur.

(ii) *Diseases of the liver*

(a) *Cirrhosis*. In this condition there is a failure to synthesize the normal amount of plasma protein. Patek and Post have suggested a high protein diet and high intake of B complex as well as carbohydrate in the treatment of hepatic cirrhosis.

(b) *Hepatitis*. The sulphur-containing amino acids, methionine and cystine, have been emphasized as the particular components of protein of importance in protecting the liver from toxic hepatitis. Whilst this may be true in animals who have been subjected to marked under-nutrition and then rendered toxic by chloroform, it is doubtful whether methionine or cystine are protective to the liver in infective hepatitis when the diet prior to the onset of the disease has been normal.

(iii) *Diseases of gastro-intestinal tract*

In such conditions as peptic ulcer, the intake of protein may be below normal and hypo-albuminaemia may develop. Ulcerative colitis is associated with much loss of blood protein from the wall of the colon and the blood serum level may be low.

(iv) *Famine oedema*

During the war gross under-nutrition was extremely common, both in the Far East and in Europe. The protein intake was reduced to a few grams per day, and the total calories to less than 1,000. In such circumstances, synthesis of blood plasma protein was deficient, and hypo-albuminaemia with subsequent oedema developed. Similar oedema will develop in any prolonged illness if the protein intake is grossly deficient.

## (2) SURGICAL CONDITIONS

(i) *Burns*

Seepage of plasma from the denuded areas is associated with a decrease in both the volume and the protein con-

centration of the plasma. In consequence, the plasma proteins fall while the haemoglobin rises.

In some cases during the first hours a loss of water from the blood occurs to such an extent that the plasma proteins show a transitory rise. This, however, is followed by a fall as the effects of protein loss accumulate. The occasional initial dehydration of the blood seems to be due partly to the passage of water from blood to tissues, as it may occur when there is no marked external loss of fluid, as by vomiting and sweating. The haemoglobin rises during this stage, as well as during the subsequent stage when the effects of seepage dominate.

(ii) *Post-operative conditions*

In the post-operative period, dehydration of the blood is likely to increase both the plasma protein and the haemoglobin concentration. The dehydration may go so far as to produce uraemia. Repeated saline injections may be required to replace fluid. However, if too much saline is administered by unregulated infusions, haemodilution and a water-logged, oedematous state of the tissues may result, which is as undesirable as the dehydration. If changes in blood gravity are followed, infusion can be so regulated that error in either direction is avoided.

Slow healing of wounds may occur if hypoproteinaemia exists. Local oedema at the site of an anastomosis, e.g. gastro-jejunostomy, may obstruct the stoma if the blood plasma protein is unduly low.

(iii) *Traumatic shock*

Varying degrees of dehydration, of plasma protein seepage from injured vessels, tissues, and surfaces and of internal and external haemorrhage can, combined, produce such unpredictable effects on blood volume and concentration that observations on the blood are especially needed, together with careful interpretation. In most cases of traumatic shock, with haemorrhage, the degree of shock has been found by Evans to be roughly proportional to the volume of blood lost, clinical shock being usually apparent when loss exceeds 15 per cent of

the normal volume, and so severe that the patient may not survive if the loss exceeds 45 per cent.

Fall of haemoglobin, during the hours after trauma, to a level below normal affords presumptive evidence of haemorrhage.

However, observation of normal haemoglobin concentration during this period by no means excludes the possibility of severe blood losses. Post-haemorrhagic entrance of fluids into the circulation may be so slow that blood gravity does not fall below normal for hours; this is likely to be the case when the patient is dehydrated, as may occur with wounded. When clinical and vascular signs of shock are present after trauma, severe external or internal haemorrhage is to be suspected, even if the blood gravity and haemoglobin concentrations are normal. The circulation volume of the blood is then low, despite the normal concentration, and immediate infusion of blood or plasma is indicated.

During the days after severe trauma destruction of red cells may set in, as after burns. This appears particularly likely to occur if fractures are involved. Anaemia may develop rapidly, and may require repeated transfusions. In such cases daily estimations of haemoglobin are desirable. To govern transfusions, the practical rule has been found that 500 ml. of blood given to an adult raises haemoglobin by 1 g. per 100 ml. (Phillips, *et al.*)

### *Estimation of "Acid" Phosphatase in Blood Serum* (Gutman and Gutman)

*Introduction.*—Kutscher and Wolbergs discovered that human prostatic tissue is extremely rich in an "acid" phosphatase. It has an optimal activity between approximately pH 4.5 and 6. The concentration of this "acid" phosphatase in adult human prostate tissue is very high—500 to 2,500 units of activity per gram of fresh tissue. Kidney, liver, duodenal mucosa and bone have an activity of less than 5 units per gram at a similar pH (4.9). "Acid" phosphatase does not appear in high concentration in human prostatic tissue until puberty. The enzyme normally is excreted in the prostatic fluid where it varies in concentration

from 700 to 3,700 units per ml. The precise physiological role of prostatic "acid" phosphatase in the semen is unknown. In 1936 Gutman, Sproul and Gutman demonstrated that large amounts of this enzyme were present in carcinomatous prostatic tissue; not only in the primary tumour but also at the site of distant metastases. It was later shown that an increase of "acid" phosphatase occurred in the blood serum of patients with metastasizing carcinoma of the prostate. This observation now serves as the basis of a valuable diagnostic procedure, as it has been found that the rise in "acid" phosphatase content of serum is almost entirely confined to patients suffering from metastases arising from carcinoma of the prostate. Gutman and Gutman have adapted the King and Armstrong method for "alkaline" phosphatase to the estimation of serum "acid" phosphatase activity.

*Principle.*—Sodium phenylphosphate is hydrolyzed by "acid" phosphatase in an acid medium and the amount of phenol liberated is estimated colorimetrically.

*Reagents.*—1. Buffer-substrate (0.005M monophenyl phosphate), (p. 365).

2. Phenol reagent of Folin and Ciocalteu, (p. 365).

3. Sodium carbonate (20 per cent solution).

4. Standard phenol, (p. 365).

5. Standard phenol solution and reagent, (p. 365).

*Procedure.*—Two test tubes each containing 10 ml. buffer-substrate solution are kept in a water bath at 37°C for about 5 minutes or until contents attain that temperature. Pipette exactly 0.5 ml. of the serum (serum must not contain haemolyzed red blood corpuscles) to be tested into each tube, stopper, mix, and incubate at 37°C for 3 hours. Then remove the tubes from the water bath, at once add 4.5 ml. of diluted phenol reagent, mix and filter. To two control tubes each containing 10 ml. of buffer-substrate solution add 0.5 ml. of the serum and at once add 4.5 ml. of diluted phenol reagent, mix and filter.

Pipette 10 ml. of each of the test and control filtrates into test-tubes, add 2.5 ml. of 20 per cent sodium carbonate solution, and mix. To 10 ml. standard phenol solution and phenol reagent (No. 5) prepared shortly before, add 2.5 ml. of 20 per cent sodium carbonate solution and mix. Place test, control and standard tubes together in the water bath at 37°C for 20 minutes to

TABLE XXX

Percentile Distribution of Serum "Acid" Phosphatase Values in Normal Subjects and in Patients with Disease of the Prostatic Gland

	Total no. of cases	Percentage of cases with serum "acid" phosphatase					
		Less than 3.0 units per 100 ml. serum	3.0 to 4.9 units	5.0 to 9.9 units	10.0 to 19.9 units	20 to 100 units	100 to 1,000 units
1. Normal subjects .. .. .	30	100%					More than 1,000 units
2. Diseases of the prostate gland .. .. .	285						
(a) prostate carcinoma; bone meta- stases (x-ray) .. .. .	130	15%	12%	25%	16%	19%	9%
(b) prostate carcinoma; no bone meta- stases (x-ray) .. .. .	70	89%	11%				4%
(c) benign prostatic hypertrophy .. .. .	75	100%					
(d) prostatitis .. .. .	10	100%					

(Sullivan, Gutman and Gutman)

phoric esters have been known for many years. In 1908 Harden and Young demonstrated that yeast juice contained an enzyme (hexose phosphatase) which was capable of hydrolysing a hexose phosphate. Since then the phosphatases have been found in many organs, particularly in ossifying cartilage, intestinal mucosa, prostate, in the cortex of the kidney and in secretory tissue of the mammary gland. In the foetus of mammals, growing bone and tooth are extremely rich in these enzymes. It seems that the phosphatases found in bone, kidney, intestine, prostate and blood plasma have the power of hydrolysing a very great variety of phosphoric esters, e.g., hexose and triose phosphates, phospholipids, nucleotides, creatine phosphate and many others. Those phosphatases which act on mono-phosphoric esters may be classified into "alkaline" and "acid" phosphatases, so called because they show optimum activity on the alkaline or acid side respectively of neutrality. The optimum activity of "alkaline" phosphatase is usually shown in a medium of approximately pH 9. It might also be noticed that the enzyme shows high activity at lower pH values when the substrate concentration is reduced. Magnesium (in traces) has a profoundly favourable influence on the enzyme's action, i.e. it acts as a co-enzyme or activator. Alkaline phosphatase occurs in the periosteum of long bones as well as in the epiphyseal regions and is also found in "membrane" bones. The cartilagenous patella before ossification has commenced contains no alkaline phosphatase, but the enzyme appears when ossification is initiated. Red blood corpuscles have within their substance a considerable amount of acid soluble phosphoric ester which can act as a suitable substrate for alkaline phosphatase, but this substrate does not seem capable of diffusing out of the intact erythrocyte. There is, however, a small quantity of acid soluble phosphoric ester in blood plasma. As has been mentioned, ossifying cartilage, e.g. in the epiphyseal regions of bone, is very rich in alkaline phosphatase and this liberates phosphoric acid from the phosphoric acid esters of the blood plasma circulating through the ossifying areas. According to Robison the local increase of concentration of phosphoric acid then causes deposition of calcium phosphate. In view of the fact that alkaline phosphatase of bone has been shown to have both synthetic as well as hydrolytic powers it is

possible, as has been suggested by Kay, that "the enzyme plays an active part also in bone resorption and demineralization under both normal and pathological conditions by synthesizing from some of the soluble calcium phosphate of the bone soluble phosphoric esters of calcium." It is probable that the increase of blood phosphatase is not a cause of the bone diseases in which it is found in excess, but rather it is an effect of the bone disease. It may be that it leaks out of the diseased bone. Normally plasma contains weight for weight only about one hundredth of the quantity found in an average specimen of adult human long bone and a slight increase of the rate of diffusion from bone into plasma could easily account for the high figures obtained in some bone diseases.

It is probable that acid soluble organic phosphorus compounds of the plasma may be the source of the inorganic phosphate of the urine, the kidney phosphatase liberating the inorganic phosphorus from the organic esters brought in the blood to the kidneys.

*Principle.*—Sodium phenyl phosphate is hydrolysed by phosphatase and the amount of phenol liberated is estimated colorimetrically.

*Reagents.*—1. Buffer substrate (p. 363).

2. Phenol reagent (p. 364).

3. 20 per cent solution of sodium carbonate (anhydrous).

4. Stock standard phenol (p. 364).

5. Standard phenol solution and reagent (p. 364).

#### *Procedure*

*Test.*—Into two test tubes introduce 10 ml. of buffer substrate. Place the tubes in a water bath at 37.5°C for five minutes or longer. With the tubes still in the bath add 0.5 ml. of serum (cell free) or plasma to each. Stopper the tubes, mix the contents and allow them to remain in the bath for exactly 15 minutes. At the end of this period add immediately 4.5 ml. of dilute phenol reagent, mix and filter at once.

*Control.*—Introduce 10 ml. buffer substrate into each of two test tubes and add to each tube 0.5 ml. of serum and 4.5 ml. diluted phenol reagent immediately, and then filter.

10 ml. of the filtrate from the test and control solutions are

pipetted into clean test tubes. 2.5 ml. of 20 per cent sodium carbonate are now added, mixed and the tubes replaced in the water bath to develop the maximum blue colour (usually in about 20-30 minutes).

*Standard.*—Introduce 10 ml. of standard phenol and reagent (No. 6) into a test tube and add 2.5 ml. of 20 per cent sodium carbonate. Immediately place in water bath with test and control.

*Comparison.*—Set the unknown at 30 and note the reading of standard when the colours match. Replace the unknown by the control and note the reading of standard when the colours match.

*Calculation.*—The phosphatase activity of serum is expressed as units per 100 ml. of serum and is numerically equal to the mg. of phenol which would be set free from the phenyl phosphate under the standard conditions by 100 ml. of serum. Thus:

Units of phosphatase per 100 ml. =

$$\left( \frac{\text{mg. phenol per 100 ml.}}{\text{serum in test}} \right) - \left( \frac{\text{mg. phenol per 100 ml.}}{\text{serum in control}} \right)$$

The number of mg. phenol in 100 ml. of serum in the test and in the control is found by the equation:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{Strength of standard} \times \frac{15}{10} \times \frac{100}{0.5}$$

With the unknown solution set at 30 mm. and the strength of standard = 0.1 mg. this equation can be written:

$$\frac{\text{Reading of standard}}{30} \times 0.1 \times \frac{15}{10} \times \frac{100}{0.5}$$

All figures in the above equation cancel out, making it equal to "Reading of standard".

More simply, then:

Units of phosphatase per 100 ml. =

$$\text{Reading of standard (against the test)} \text{ minus Reading of standard (against the control)}$$

*Procedure when the enzyme content of the serum is very high.*—According to King and Armstrong if more than 30 units are present but less than 60 units the reading of the unknown for the test solution should be set at 15 mm. In such a case "Reading of standard" (against test) must then be multiplied by

two to obtain mg. phenol per 100 ml. of serum. For routine work it is their custom to dilute high phosphatase sera with normal saline so that the number of units per 100 ml. of diluted serum will not be in excess of 60.

*Clinical value of estimation of "alkaline" phosphatase in blood serum.*—Most of the "alkaline" serum phosphatase is derived from bones, whence it escapes into the blood to pass through the liver and be excreted in the bile. The phosphatase activity of the serum is increased chiefly in two types of disease: (a) in certain disorders of the bone in which more enzyme is formed, and (b) in certain disorders of the liver biliary tract in which the excretion of bile is interfered with, and the enzyme tends to accumulate in the blood. The latter circumstance can be utilized to differentiate between obstructive and hepatogenous jaundice, because, in general, occlusion of the intra- or extra-hepatic biliary tract causes larger amounts of phosphatase to appear in the blood than are associated with inflammatory or degenerative changes in the liver parenchyma (Gutman). Using King and Armstrong's method the normal value of "alkaline" phosphatase in blood serum lies between 3 and 13 units. A great increase in obstructive jaundice (65 to 162 units) occurs. The increase in obstructive jaundice was confirmed experimentally by ligating the common bile duct in dogs which caused the "alkaline" phosphatase content of the blood serum to increase 30 to 100-fold the initial amount.

Kay, using an older technique, has found that in certain generalized diseases of bone—osteitis fibrosa, osteitis deformans, osteomalacia and rickets—the plasma phosphatase is increased.

In an analysis of 116 cases of Paget's disease (osteitis deformans), Gutman and Kasabach found the serum calcium and inorganic phosphorus were within normal limits, but the blood "alkaline" phosphatase was almost invariably increased. In many cases of Paget's disease there was a constancy of the high serum phosphatase level when estimated at intervals. The blood phosphatase activity was roughly proportional to the extent and activity of the lesions in this disease. In hyperparathyroidism there is as a rule hypercalcaemia, hypophosphataemia and increased blood phosphatase with figures of 100 units or more. In some cases the blood calcium is not raised and in others the

blood phosphorus is not lowered. Gutman and his colleagues showed that approximately 80 per cent had hypercalcaemia with a calcium content of more than 12 mg. per 100 ml. of blood. About 50 per cent showed hypophosphataemia with an inorganic phosphorus of less than 2.5 mg. Increase in phosphatase was found in practically all cases.

Smith and Maizels demonstrated that during the treatment of rickets the plasma phosphatase fell from a high figure to normal; on the other hand, in scurvy normal figures were found during the acute haemorrhagic stage and a marked rise in blood phosphatase occurred when calcification set in. These authorities point out that the phosphatase in rickets remains high long after the serum calcium and the inorganic phosphate have returned to normal and they suggest that the plasma phosphatase is probably a more delicate test of calcium and phosphorus metabolism than any other yet used.

Table XXXI indicates the average findings of calcium, phosphorus and phosphatase in some pathological conditions.

**TABLE XXXI**  
**Calcium, Phosphorus and Phosphatase in some Pathological Conditions**

<i>Nature of Disease</i>	<i>Blood Chemistry</i>		
	<i>Ca</i>	<i>Inorganic P</i>	<i>"Alkaline" Serum Phosphatase</i>
Hyperparathyroidism	Increased	Diminished	Increased
Paget's Disease	Normal	Normal	Increased
Multiple Myeloma	Increased	Normal	Normal
Metastatic carcinoma of bone	Increased (occasionally)	Normal	Normal (?)
Chronic renal lesions	Normal or diminished	Raised	Normal (?)
Rickets	Diminished (slightly)	Diminished	Increased

of infection of the meninges and in space occupying lesions inside the cranium.

Table XXXIII indicates the average findings of calcium, phosphorus and cerebro-spinal fluid in regard to certain chemical constituents of clinical interest.

TABLE XXXIII  
Compositions of Plasma and CSF compared

Constituents	Mg. per 100 ml.		m-Eq. per litre	
	Plasma	C.S.F.	Plasma	C.S.F.
Na .. ..	325	325	141	141
K .. ..	20	10	5	2.5
Ca .. ..	10	5	5	2.5
Mg .. ..	3	3	2	2
<b>Cations</b>	—	—	153	148
Cl .. ..	360	449	101	126.5
HCO <sub>3</sub> (vols. CO <sub>2</sub> ) ..	60	40	27	18
P .. ..	3	2	2	1
S .. ..	1	0.5	1	0.5
Organic acids .. ..	traces	traces	2	2
Proteins .. ..	7,000	30	20	negligible
<b>Anions</b>	—	—	153	148
Urea .. ..	30	24	5	4
Sugar .. ..	90	72	5	4
<b>Totals</b>	—	—	316	304

(from Harrison, *Chemical Methods in Clinical Medicine*)

Two outstanding features in this table are the difference in protein and chloride content of the two fluids. The low osmotic pressure due to deficiency of proteins in the cerebro-spinal fluid is more than balanced by the increase in chloride; but glucose, cholesterol, uric acid and many other components of plasma found in greater concentration in this fluid than in cerebro-spinal fluid help to offset the osmotic effect of the chloride so that actually the two fluids are iso-osmotic.

**Formation.**—Cerebro-spinal fluid is formed by the choroid plexus. The fluid passes by the foramina of Munro into the third ventricle and thence by the Sylvian aqueduct to the fourth ventricle, from which it emerges by the foramina of Majendie and

Luschka into the subarachnoid space. That the choroid plexus is the site of formation of the cerebro-spinal fluid is indicated in various ways. Perhaps the most striking experimental proof is afforded by occlusion of one foramen of Munro, which induces unilateral hydrocephalus. This does not occur if the choroid plexus of the corresponding lateral ventricle has previously been removed.

Whether the formation is a true secretion as Dixon, Halliburton and other investigators maintained, or whether it is a dialysate produced by filtration through a semi-permeable membrane is still undecided. Colloids are held back by the choroid plexus and, although an increase of glucose or urea in the blood is followed by a corresponding rise in concentration of these crystalloids in the cerebro-spinal fluid, a simple molecule such as that of sodium iodide does not pass the choroid plexus, the cells of which seem to have a selective permeability.

*Haemato-encephalic barrier.*—This title has been given by Stern and Gautier to the barrier between blood and cerebro-spinal fluid. There is much evidence to indicate that the choroid plexus protects the cerebro-spinal fluid from changes in the composition of the blood.

Various physiological and pathological factors influence the function of the barrier between blood and CSF:

(a) In newborn animals (kitten, puppy) crystalloids such as sodium iodide and bismuth subnitrate pass readily from the blood into the CSF, but as the animal grows older the barrier resistance gradually increases until it becomes impervious to these substances.

(b) A rise in body temperature is stated to increase the barrier permeability.

(c) Inflammatory processes in the cerebro-spinal system may considerably increase the passage of substances from blood to CSF. Aseptic meningitis induced by injections intraspinally of serum, salvarsanized serum or a solution of caseinogen have been followed by increased permeability of the haemato-encephalic barrier.

Under normal conditions, arsenic does not pass readily from the blood into CSF, but according to Katzenelbogen the passage of this substance is greatly increased in neuro-syphilis. This is

of infection of the meninges and in space occupying lesions inside the cranium.

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Under normal conditions, arsenic does not pass readily from the blood into CSF, but according to Katzenelbogen the passage of this substance is greatly increased in neuro-syphilis. This is

probably true also of bismuth compounds. There is much conflicting evidence concerning the passage of antibodies from the blood into the CSF. It is apt to vary considerably, but as a rule is not marked even when the antibody titre in the blood is high. Attempts have been made to increase the passage of substances from the general circulation to the CSF by draining off fluid by lumbar puncture. Results on the whole have been disappointing.

*Absorption.*—The absorption of cerebro-spinal fluid from the sub-arachnoid space occurs mainly by filtration through the arachnoidal villi into the dural venous sinuses. In the cat the pressure in the venous sinuses varies from 77 to 115 mm. of water, whereas the pressure in the subarachnoid space is higher, being 120 mm. water, and thus induces filtration.

The normal pressure of the cerebro-spinal fluid is 110 to 130 mm. of Ringer's solution for man in the recumbent position. Posture greatly alters the figure, the pressure in the lumbar region is approximately 200 mm. higher in the sitting than in the prone position.

Weed and McKibben demonstrated that the pressure of cerebro-spinal fluid can be markedly influenced by the intravenous injection of hypertonic saline or glucose. If 100 ml. of hypertonic saline (15 to 30 per cent NaCl), or 100 ml. of 50 per cent glucose dissolved in normal saline, be injected intravenously, a marked, but temporary, fall in cerebro-spinal pressure and shrinkage in brain volume occurs. In these circumstances the absorption is due to the increased osmotic pressure of the blood plasma. There is some evidence that under these conditions the absorption of the fluid is largely by way of the perivascular spaces. This method of lowering intracranial tension has been used in the treatment of intracranial tumours, persistent cerebral contusions and other cerebral conditions.

It is doubtful if absorption normally occurs by way of the perivascular (Virchow Robin) spaces, as the flow along these channels is normally towards the sub-arachnoid space, into which they carry the waste products of cellular metabolism. The circulation of the fluid from its site of formation to its exit by absorption into the venous system is facilitated by waves imparted to it by arterial and venous pulsations. Cerebro-spinal fluid and venous blood are driven out of the cranium with each

arterial pulse, some of the fluid being forced into the sub-arachnoid space of the spinal column. Forced expiration, such as coughing or crying, markedly increases the cerebro-spinal pressure.

In man with a vertical spinal column there is considerable difficulty in maintaining a circulation from its caudal to its cranial end against gravity. It is facilitated by a plexus of veins in the space between the dura mater and the laminae arches of the dorsal and lumbar regions, which becomes distended during expiration and drives the fluid towards the cervical regions of the cord. There is, however, a tendency in pathological conditions for blood corpuscles, pus cells, and other cellular material to sediment at the lower end of the canal, so that fluid obtained by lumbar puncture at this level may be much richer in cell content than that at the foramen magnum.

Apart from clinical examination of the patient three methods are available for the detection of obstruction of the spinal sub-arachnoid space: radiography, manometry, and chemical examination of the cerebro-spinal fluid.

(a) In the first method, lipiodol, a chemical compound containing iodine bound to a vegetable oil, is introduced into the sub-arachnoid space either by cisternal puncture using heavy lipiodol when the material falls to the site of obstruction in the sub-arachnoid space or by lumbar puncture using light lipiodol when it rises to the site of obstruction. Lipiodol is opaque to X-ray and hence locates the spinal block.

Encephalography has been used in recent years to visualize the cerebral ventricular system.

(b) By means of a manometer connected with the sub-arachnoid space through lumbar puncture Queckenstedt's test may be performed. By combined cisternal and lumbar puncture differences in pressure above and below the block may be determined.

(c) Biochemical changes may occur below the lesion in spinal block and these changes in the fluid are often referred to as the "syndrome of Froin." Several factors are responsible for the change in the fluid. If the lesion responsible for the obstruction compresses the spinal veins, chronic venous congestion of the cord occurs below the obstruction and transudation of plasma

from the blood vessels follows. If the obstruction is accompanied by a localized meningitis plasma may reach the spinal fluid in the form of an inflammatory exudate. It will be readily understood then that obstruction of the spinal sub-arachnoid space may lead to a great excess of protein in the fluid below the site of the lesion. Furthermore, the fluid may be yellow in colour (xanthochromia) and may coagulate spontaneously on standing. Occasionally xanthochromia and excessive protein has been observed in the fluid drawn from above the level of a tumour causing sub-arachnoid block. This may be due to transudation of plasma from the vessels of the tumour.

## CHEMICAL EXAMINATION OF THE CEREBRO-SPINAL FLUID

### (A) QUALITATIVE EXAMINATION

#### (1) PROTEIN

In the past it was usual to lay emphasis on increase of globulin in the cerebro-spinal fluid and pay little heed to the total protein. It is now known that an increase in globulin is accompanied by an increase in albumin and in most cases the amount of albumin is in excess of globulin. An estimation of the total protein is usually of more clinical value than a test for increased globulin only.

#### *Nonne-Apelt Test*

*Principle.*—Globulin is precipitated from solution by half saturation with ammonium sulphate.

*Reagent.*—Saturated ammonium sulphate.

*Procedure.*—To 1 ml. of cerebro-spinal fluid add an equal volume of saturated ammonium sulphate. If the tube be shaken, an opalescence or cloudiness in the mixed fluids indicates the precipitation of globulin.

The second part of this test (phase two) consists in acidifying and boiling the clear fluid obtained by filtering the mixture of cerebro-spinal fluid and ammonium sulphate. A precipitate indicates the presence of albumin.

*Clinical value of qualitative test for protein:* Normal cerebro-spinal fluid remains clear with this test. In meningo-vascular syphilis, tabes and G.P.I. there may be marked turbidity in

phase one indicating increase of globulin and in pyogenic meningitis phase two shows great increase in albumin. Quantitative estimations are of greater importance.

## (2) GLUCOSE

### *Benedict's Test*

To 1 ml. of CSF add 1 ml. of Benedict's qualitative reagent. Boil for two minutes and allow to cool. An opalescent greenish supernatant fluid with a slight yellow precipitate occurs in normal fluid.

*Clinical value of qualitative test for glucose.*—In suppurative meningitis the sugar may be markedly diminished or absent. In diabetes there is a rise in the sugar content of CSF which follows the sugar percentage in the blood.

## (B) QUANTITATIVE EXAMINATION

For clinical purposes estimation of sugar, chloride or protein may be indicated.

### (1) SUGAR

The method of Hagedorn and Jensen is performed exactly as for blood (see p. 95).

*Clinical value of quantitative sugar estimation.*—In table XXXIV it will be noted that in meningitis due to a pyogenic infection glucose is markedly diminished or even absent from the CSF owing to the fact that the micro-organisms have caused its destruction by fermentation. In tuberculous meningitis there is also a diminution in the glucose content of CSF which is not so marked as in pyogenic infections, but virus meningitis usually causes little or no alteration of the glucose of the CSF. In diabetes mellitus it may be markedly increased in quantity.

### (2) CHLORIDES

*Principle.*—When silver nitrate is added to a chloride solution, silver chloride is precipitated and the end point is detected in the presence of potassium chromate by the development of a permanent faint red colour.

*Reagents.*—(a) Silver nitrate (5.814 gm. per litre).

(1 ml.  $\text{AgNO}_3$  = 2mg.  $\text{NaCl}$ )

(b) Potassium chromate (10 per cent).

*Procedure.*—Introduce 1 ml. CSF into a porcelain dish with 2 drops of potassium chromate as indicator and from a micro burette run in silver nitrate till a permanent red colour occurs.

*Calculation.*—Titration in ml.  $\times 2 \times 100 =$  mg. chloride per 100 ml. of CSF.

*Clinical value of chloride estimation.*—The normal amount of chloride (expressed as NaCl) is 700 to 760 mg. per cent. In tuberculous meningitis the figure may be reduced to 550 mg. or less, but it usually ranges between 550 and 650 mg. In meningococcal, pneumococcal and staphylococcal meningitis the figure usually lies between 600 and 750 mg.

TABLE XXXIV  
The CSF in Meningitis

	<i>Pyogenic</i>	<i>Tuberculous</i>	<i>Virus</i>
Pressure	Raised	Raised	Slight increase
Appearance	Turbid	Clear or slightly opalescent	Usually clear
Cells	Polymorphs, very numerous	Usually more lymphocytes than polymorphs	Lymphocytes
Protein	100 to 200 mg. or more/100 ml.	100 to 200 mg./100 ml.	Up to 150 mg./100 ml.
Globulin	Positive	Positive	Positive
Chlorides	About 650 mg / 100 ml.	550 to 650 mg./100 ml.	Slight decrease
Sugar	Trace or absent	Decreased to 20 to 30 mg./100 ml.	Usually normal

### (3) PROTEIN

Method of Exton applied by Beaumont and Dodds to cerebro-spinal fluid.

*Principle.*—The turbidity produced by precipitating the protein in a known volume of cerebro-spinal fluid is compared with that which occurs on precipitating protein solutions of known concentration in a similar manner.

*Reagents.*—1. Exton's reagent (see p. 358).

2. Standard protein solutions (see p. 358).

*Procedure.*—In performing the test add 1 volume of Exton's reagent to 1 volume of cerebro-spinal fluid. After allowing to

stand for 10 minutes the content of the tube is compared with standard tubes. These tubes should be inverted several times to ensure thorough mixing. If the protein content of the cerebro-spinal fluid is above 0.1 per cent it is diluted suitably so that an estimation can be made.

*Clinical value of quantitative protein estimation.*—In all forms of meningitis there is a rise in the protein content of the CSF most marked in pyogenic infections in which it may be 200 mg. per cent or more. This is shown in Table XXXIV. In syphilitic conditions (Table XXXV) not only is the protein content raised but there is a relative increase of globulin.

TABLE XXXV  
The CSF in Syphilis

	<i>Meningo-vascular</i>	<i>Tabs</i>	<i>G.P.I.</i>
Pressure	Normal or slightly raised	Normal or slightly raised	Normal or slightly raised
Appearance	Clear and colourless	Clear and colourless	Clear and colourless
Cells	Mostly lymphocytes	Lymphocytes, a few large mono-nuclears	Usually lymphocytes and large mononuclears
Protein	Usually up to 100 mg./100 ml.	Usually up to 100 mg./100 ml.	50-150 mg./100 ml.
Globulin	Positive (+)	Positive (++)	Positive (++)
Lange test	Usually mid-zone	Leutic, (0012332100)	Paretic, (5544322110)
Wasserman reaction	+	Usually ++	+++

### *Lange Colloidal Gold Test*

*Principle.*—Gold is precipitated from colloidal suspension by globulin in pathological cerebro-spinal fluid and the colours are used in diagnosis.

*Procedure.*—(For full details the reader is referred to larger text books of clinical biochemistry).

### REFERENCES

## XX

### EXAMINATION OF FAECES

#### INTRODUCTION

Faeces consist of the undigested and indigestible residue of substances taken in the food, together with numerous bacteria and the more or less changed secretions and excretions which are passed into the alimentary canal. Diet has a very marked influence on the appearance of the faeces and in examining them in pathological conditions it is essential to have a fixed diet. Many diets have been suggested, and of these perhaps the most suitable is the Hawk intestinal diet, which is as follows:

- Breakfast: 100 grams oatmeal  
60 grams toast  
20 grams butter  
250 ml. milk
- Luncheon: Rice soup (chicken broth with rice)  
100 grams green vegetables (asparagus)  
100 grams mashed potatoes  
60 grams toast  
20 grams butter  
250 ml. milk
- 4 o'clock: 250 ml. milk
- Dinner: 150 grams chopped meat, grilled on the outside  
and raw in the centre  
100 grams green vegetables (spinach)  
100 grams mashed potatoes  
60 grams toast  
20 grams butter  
250 ml. milk  
Stewed fruit

The test diet is to be given for three days, or longer, until a motion is obtained which comes with certainty from the diet. This can generally be recognized by its uniform consistence and lighter colour; usually the second or third defaecation will show

it. Powdered charcoal or carmine (0.2 to 0.5 g. of either) in cachet will colour the faeces grey or red, and will, if given at the beginning and end of a test period, serve to accurately demarcate the period in question. Faeces may be collected in glass jars with clamped tops, and may readily be handled with a wooden spoon or spatula. The examination should be made as soon as possible after evacuation, but if there is delay the material may be kept in a refrigerator or formalin may be used as a preservative if the material has to be sent considerable distances. The faeces may be dried on a water bath, powdered and stored for future analyses.

## GENERAL CHARACTERISTICS

### (1) INTRODUCTION

The influence of bile and of the secretions of the stomach (chap. i) and the pancreas (chap. v) on the process of digestion has been already discussed. It is now opportune to mention the nature and action of the intestinal secretions. These have been investigated by Florey, Wright and Jennings.

(a) *Duodenal juice*.—It is chiefly secreted by Brunner's glands, but some part may be played by the villi and crypts of Lieberkühn. It is rich in mucin, which is not precipitated by acid, and normally protects the mucous membrane from the action of hydrochloric acid squirted through the pylorus into the duodenum by the peristaltic action of the stomach. The only enzymes constantly present are amylase and enterokinase.

(b) *Intestinal juice*.—Enterokinase is the most important constituent of the secretion of the small intestine. Amylase is a normal component, but invertase, lactase, peptidase, lipase, phosphatase and probably maltase are derived from cast off epithelial cells of the intestinal mucous membrane, and are not constituents of the actual intestinal secretion. They exert their digestive function on the appropriate substrate during its absorption through the intestinal epithelium. The phosphatase of the intestinal mucosa acts upon nucleotides, glycerophosphates and hexosephosphates, liberating inorganic phosphate and the organic component—nucleosides, glycerol or hexose respectively. A single phosphatase is involved in these reactions. Water and salt solutions are readily absorbed by the small intestine, and it

has been suggested (Wright *et al.*) that "it may be necessary for a constant secretion of fluid to take place from the crypts of Lieberkühn to keep food particles in suspension while they are attacked by the pancreatic enzymes, and as the products of digestion are absorbed, water and salts go with them. One may therefore envisage a circulation of fluid during active digestion, the secretion passing out from the crypts of Lieberkühn into the intestinal lumen and back into the villi."

(c) *Caecum and Colon*.—The secretion, which may be markedly alkaline (pH 8.3 to 8.4 on collection), contains no enzymes except perhaps dipeptidase and a trace of amylase. It is very rich in mucus, which lubricates the faeces. If the faeces contain irritant matter extra fluid is secreted to dilute the irritant and the mucoid fluid prevents its rapid diffusion. The alkali in the secretion neutralizes acids produced by action of bacteria on the intestinal contents. Barbiturates markedly reduce and almost abolish the secretion of the large intestine. Various investigators in recent years have supplied little evidence of excretion of calcium or phosphorus by the large intestine except in minimal amounts. Leitch could not find satisfactory evidence that mercury, bismuth or organic arsenical compounds were excreted by the colon as has been thought in the past. Much water is absorbed in the colon.

The following approximate quantities of fluid are secreted into the alimentary tract each 24 hours.

Saliva	1,500 ml.
Gastric secretion	2,500 ml.
Bile	500 ml.
Pancreatic juice	700 ml.
Intestinal secretion	3,000 ml.
	<hr/>
Total	8,200 ml.

Practically all this is reabsorbed leaving only about 100 ml. to be excreted in the faeces.

## (2) QUANTITY

This varies greatly with the diet, but 100 to 150 g. per day may be taken as an average figure, but is much increased on a

vegetable diet, being 350 g. or more whereas on a meat diet the amount of faeces may be only 50 to 60 g. Infants and children pass relatively less faeces than adults. Pathologically the amount in adults may be 500 to 1,000 g. or more per diem. Faeces are still excreted from the intestine when no food is ingested, usually 10 to 20 g. per diem.

### (3) COLOUR

On a mixed diet, the colour is usually light brown or greenish brown, due to the presence of stercobilin which is formed from bilirubin by reduction in the intestine, largely as the result of bacterial activity. The faeces of infants are usually yellow, due to milk diet, and to unchanged bilirubin. Special articles of diet may affect the colour. Pathologically the colour may be green, as occurs in diarrhoea of children, and is due to unchanged biliverdin and the activity of chromogenic bacteria. If the bile duct is obstructed, very pale motions are obtained. Bismuth and iron give rise to black faeces and calomel may cause them to become green, due to the presence of biliverdin. Large amounts of changed blood, resulting from haemorrhage in the upper alimentary tract, cause black tarry motions. If the

TABLE XXXVI

Influence of some Foods and Drugs upon the Colour of the Faeces

Milk	White or pale orange
Meat	Brownish black
Chlorophyllaceous vegetables	Greenish
Non-chlorophyllaceous vegetables	Light brown
Cherries or blackberries	Reddish brown
Cocoa	Dark red or red brown
Coffee	Dark brown
Bismuth subnitrate	Black
Calomel	Green
Iron	Black—on exposure to air
Methylene blue	Blue—on exposure to air
Senna	Dark yellow
Santonin	Dark yellow
Rhubarb	Yellow

haemorrhage is in the vicinity of the rectum, the blood appears bright red.

Table XXXVI (modified from Hawk and Bergeim) briefly summarizes several of these facts.

#### (4) CONSISTENCY

Both in health and disease great variation in consistency occurs. Scybalous masses are usually present in habitual constipation. Ribbon-like stools are suggestive of obstruction in the rectum, due to carcinoma or syphilitic stricture. Liquid stools occur in various forms of enteritis and colitis. Mucus in excess occurs in mucomembranous colic and in colitis.

#### (5) ODOUR

This is mainly due to skatol and indol, derived from the putrefaction of proteins, and is therefore most marked on a diet rich in meat. A slightly sour odour, due to fatty acids, is normal in nursing infants. Very foul-smelling faeces in adults is suggestive of malignant or syphilitic ulceration of the rectum.

### (A) MACROSCOPIC EXAMINATION

Macroscopic examination is most important, as it determines whether colour, consistence and odour correspond to normal faeces. Stir up the whole mass of faeces and transfer a piece the size of a walnut to a mortar; grind up with a little distilled water to a thin paste. This must be done carefully and thoroughly. Now spread the material over a flat glass plate in as thin a layer as possible, and examine carefully.

#### (1) PHYSIOLOGICAL

Normally if the Hawk meals be given we find chaffy remains of the oatmeal gruel, the nature of which is ultimately explained by microscopic examination; otherwise, it is entirely homogeneous.

#### (2) PATHOLOGICAL

1. Remains of connective tissue and tendon from the chopped meat. They are pale yellow, thread-like and solid. Isolated little tendon fibres are occasionally found with normal digestion, but if numerous and large, they denote disturbance of gastric digestion.

2. Remains of muscle tissue in the form of small brown-coloured rods, like splinters of wood, which show striated muscular structure under the microscope. Very much muscle points to a disturbance of intestinal digestion.

3. Occasionally 1 and 2 occur combined.

4. Potato remains, which appear as glassy, transparent granules easily mistaken for flakes of mucus. Microscopically, they show potato cells with remains of starch granules which may stain blue with iodine.

5. Fat which is light in colour and clay-like consistency occurs in excess in obstruction of the pancreatic duct. Fat is soluble in ether and stains with Sudan III. Large quantities of fatty acids appear in the faeces when there is obstruction of the hepatic or common bile ducts.

6. Mucus is continually being secreted by the healthy mucus membrane of the alimentary tract, but not in quantities sufficient to be obvious to the naked eye. Pathologically mucus in large and small flakes can be easily recognized if some of the ground-up mass is placed on a glass plate and held up to the light. It appears as glassy, transparent flakes occasionally coloured yellow by bilirubin and with irregular ragged outline. When the mucus is small in amount and intimately mixed with faeces, the lesion is probably in the small intestine. Large amounts, not well mixed with the faeces, occur in inflammation of the large intestine. Dysentery and colitis are associated with much mucus in the stools.

7. Gall stones are recognized by their faceted surfaces, or, if these be absent, by their solubility in alcohol with deposition of cholesterol crystals when the alcohol is evaporated.

8. Blood may cause the stools to be red if the haemorrhage is near the anus. If the site of the haemorrhage is high in the alimentary tract, then the motions are tar-coloured or black, owing to altered blood. If red corpuscles can be recognized microscopically, then the source of the bleeding is in the colon, rectum, or anus, but if the haemorrhage occurs high in the intestine, the red cells are disintegrated and chemical tests must be applied to detect the presence of blood. If blood, with or without mucus is proved to be in the stools, it is an indication for

further investigation by digital rectal examination, sigmoidoscopy, barium enema, or opaque meal.

9. Pus in faeces occurs in suppurative conditions of the bowel or when an abscess opens into the bowel. If the site of suppuration is high in the digestive tract, much of the pus may have become digested before reaching the rectum.

10. Portions of tape worm may be seen and must be distinguished from vegetable fibres occurring as an indigestible residue.

11. The faeces of infants frequently contain curd-like masses, due to imperfect digestion of caseinogen or fat, or excess of these in their diet. If due to fat, the masses are soluble in ether and stain with Sudan III. Casein (Ca caseate) when dissolved in 10 per cent caustic soda will give the biuret or Millon's test for protein.

12. Large crystals of ammonium-magnesium-phosphate may occur, associated with intestinal fermentation.

### (B) MICROSCOPIC EXAMINATION

Any suspicious or unusual particle is placed upon a slide and thinned with water if necessary, then covered with a cover glass and examined microscopically. It is wise to make three preparations on microscope slides as follows:

(1) A layer of the thin watery paste covered with a cover glass.

(2) A drop of the paste mixed with 30 per cent acetic acid, heated for a moment over a flame, then covered with a cover glass.

(3) A small portion mixed with a drop of Lugol's solution and covered with a cover glass.

#### (1) PHYSIOLOGICAL

In (1) may be found:

(a) Isolated fragments of muscle fibres, yellow in colour, with rounded edges, occasionally with cross striations. If a little eosin solution is run under the cover slip, muscle fibres will take up the stain and show more distinctly.

(b) Scattered large and small flakes consisting of soap (calcium

salts of fatty acid) with irregular borders, bright and deep yellow in colour.

(c) Isolated potato cells, either empty or with remains of contents which can no longer be differentiated.

(d) Cellulose from the oatmeal gruel and granular debris which constitutes the bulk of the stool.

(e) Bacteria, which are mainly dead, constitute about one-third of the weight of the dry stool. As a rule, it is of little clinical value to identify them. When the diet is chiefly carbohydrate the field of bacteria is strongly Gram positive.

(f) Yeast cells are often present in normal faeces, and they may show budding, and form short chains. They may be very numerous in intestinal fermentation.

(g) If mixed vegetables have been allowed in the diet (Hawk's diet), then various fibres and cells will be present in the faeces. Those most frequently seen have a spiral structure, and in addition, numerous cells may be grouped to form palisade parenchyma (Fig. 36).

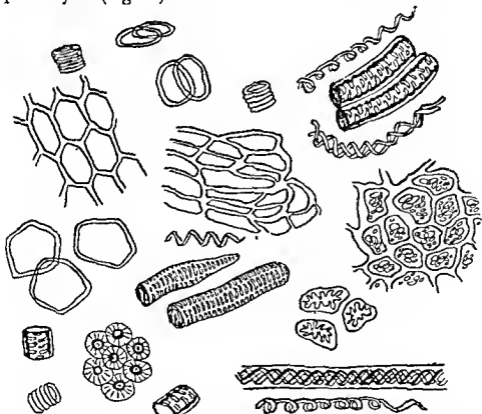


FIG. 36 Microscopic examination of faeces: vegetable fibres. (From H. Varley, *Practical Clinical Biochemistry*, Heinemann)

In (2) may be found:

Small flakes of fatty acid which, when warmed, melt into drops and solidify as they cool again.

In (3) may be found:

Potato remains which have assumed a light violet colour, due to dextrin and not to starch. The blue-coloured starch granules are absent.

## (2) PATHOLOGICAL

In (1): (a) Fragments of muscle in larger numbers than normal and better preservation, with cross striations and sharp edges, or as collections of irregularly outlined homogeneous flakes. This indicates poor pancreatic function. If the muscle fibres are still bound together by yellow elastic or white fibrous tissue, then there is a disturbance of gastric digestion.

(b) Numerous needles of fatty acid; acicular crystals, soluble in alcohol and ether and not stained by Sudan III. They may resemble bacteria, but are longer and frequently curved.

(c) Drops of neutral fat, which will stain deep orange when Sudan III. is introduced under the cover slip.

(d) Soaps.—Insoluble in ether and appearing as plaques with rolled edges or masses of acicular crystals. Soaps may be identified by mixing a small particle of faeces with two drops of copper nitrate solution on a slide and covering with a slip. On examination in a few minutes the soaps are stained green. Fatty acids are not stained. The presence of a little soap is physiological, but much is abnormal.

(e) Numerous groups of potato cells with more or less well-preserved starch granules.

(f) Mucus in excess, pus, red blood corpuscles, animal parasites, and ova.

(g) Pathogenic bacteria, e.g. *B. typhosus*, *B. dysenteriae* recognized only by culture.

In (2): Massive flakes of fatty acid. A marked increase of neutral fat, which is normally hydrolysed by the lipase of the pancreas, usually indicates blockage of the pancreatic duct or a disturbance of pancreatic function. If there be biliary obstruction without pancreatic involvement then the fats will be hydrolysed

in the usual way, but the fatty acids and soaps which are formed will not be absorbed owing to the absence of bile, and hence they will appear in large quantity in the faeces. Excessive ingestion of fat in infants may cause it to appear in considerable amounts in the faeces.

In (3): Blue-coloured remains of starch granules in potato cells, or free. This usually indicates an excessive intake or a poor digestion of carbohydrate food.

### (C) CHEMICAL EXAMINATION

#### (1) REACTION

This may be determined by putting a drop of the freshly ground-up faeces on red and blue litmus paper respectively, and examining the under surface. Normally the reaction is amphoteric or, at most, feebly acid or feebly alkaline. In infants or children on an excessive carbohydrate diet the reaction may be markedly acid, whereas an abundance of protein in the diet may cause the motion to become strongly alkaline. In some diseases in which hydrolysis of fat is normal but absorption of the fatty acid is defective the reaction is frequently acid. In addition to the influence of diet the reaction depends on the secretions into the alimentary tract, the rate of passage of the intestinal contents and on the bacterial flora.

#### (2) OCCULT BLOOD

*Introduction.*—When a small amount of bleeding occurs from a gastric carcinoma, gastric or duodenal ulcer or other lesion in the upper part of the intestinal tract, the blood becomes mixed with the food and ultimately appears in the faeces. Inspection of the faeces by the naked eye may not reveal any blood as it is intimately mixed with the food material in its passage along the alimentary tract. However, when certain chemical tests are performed, the presence of blood may be readily demonstrated. Such blood is referred to as "occult" blood. If the blood is derived from the lower intestinal tract it may be detected by inspection, as it will not have become incorporated with the faeces. The test for occult blood must never be performed unless the diet has been free from meat for at least three days. Preferably, the patient should be given a chlorophyll-free

farinaceous diet, leaving a moderately large indigestible cellulose residue, as a purely milk diet may cause the bleeding to cease temporarily, and the test become negative. For this reason, Maclean recommends that the ordinary diet, including meat, should be continued as in his experience, normal persons on such a diet do not show occult blood in their faeces. This, however, is not the case in Australia, where a normal person on a mixed diet containing meat will, in most instances, give a positive benzidine test with the boiled faeces. If the bleeding in the upper alimentary canal be slight in amount, the haemoglobin may be converted into iron-free porphyrins before reaching the rectum. This is stated to occur in as much as 16 per cent of cases of malignancy of the stomach and intestine. If the special tests for porphyrins are not performed then minute haemorrhages from these malignancies would not be detected by the ordinary test for occult blood and the underlying disease may be overlooked, for porphyrins do not give the benzidine test.

### *Tests for Occult Blood*

#### (1) BENZIDINE TEST

*Principle.*—(See p. 258).

*Reagents.*—(See p. 258).

*Procedure.*—A faecal suspension is prepared with distilled water and boiled in a test tube for three minutes to kill oxidases, and then cooled. Two ml. of a freshly prepared saturated solution of benzidine are mixed with 1 ml. of the boiled faecal suspension and 0.5 ml. of hydrogen peroxide solution (3 per cent) added. If blood be present, the solution turns blue within three minutes. A control test is performed, using water in place of the faecal extract. This test, as described, is usually all that is necessary, however, great care must be taken to use clean test tubes in performing the test, as traces of iodide or bromide may give a positive reaction in the absence of haemoglobin, but these chemicals are insoluble in ether. The oxidases present in pus or saliva, which may contaminate the faeces, are destroyed by boiling. If any doubt exists as to the accuracy of the test, it is well to add about 2 ml. of glacial acetic acid to 5 ml. of the faecal suspension and extract the acid haematin formed with 5 ml. of ether. Should the ether not separate well, add half its volume of

alcohol and mix gently, when separation should occur. Now apply the benzidine test to the ethereal extract which contains the acid haematin.

## (2) GREGERSON BENZIDINE TEST (*modified*)

Gregerson demonstrated many years ago that the sensitivity of the benzidine test depended on the concentration of benzidine in the test solution; an 8 per cent solution detecting 1:20,000 and a 0.5 per cent solution 1:500 blood in faeces.

Recently Needham and Simpson have used a modified Gregerson test. Individual powders wrapped in wax paper, each consisting of a finely divided mixture of 25 mg. benzidine hydrochloride and 200 mg. barium peroxide, are prepared. To make the test solution, shake up one of the powders in 5 ml. of glacial acetic acid. In performing the test a small quantity of faecal material is smeared on a white filter paper and a little of the benzidine solution is poured over the smear. The colour reactions have been classified as:

Positive (+); a deep blue colour appearing within 15 seconds.

Weakly positive ( $\pm$ ); a greenish-blue colour appearing within 30 seconds.

Negative (-); no coloration appearing within 30 seconds.

These investigators found the test would detect as little as 0.2 per cent (1 in 500) of blood in faeces.

By introducing blood into the stomach by means of a Ryle's tube they were able to demonstrate that it required 3 to 5 ml. to convert a negative to a positive faecal test. Of the various foods in an ordinary diet only black pudding (which contains 25 to 30 per cent of blood) or liver caused a positive modified benzidine test in the faeces. It is claimed that this test whilst avoiding oversensitivity is still sensitive enough to detect clinically significant bleeding in most instances where it is occurring.

## (3) SPECTROSCOPIC EXAMINATION FOR OCCULT BLOOD

Haemoglobin is converted into acid haematin and this is dissolved in ether and examined spectroscopically. Porphyrins, if present, are dissolved in an acid aqueous medium and examined spectroscopically.

gen and stercobilin. If the intestinal contents are hurried through very rapidly bilirubin or biliverdin may appear and respond to tests for these pigments.

### *Fouchet's Test*

*Principle.*—(See p. 47).

*Reagent.*—(See p. 47).

*Procedure.*—Prepare a suspension of faeces in water (about 1 in 10 or 1 in 20). A little of this is placed on a white porcelain tile and to this is added Fouchet's reagent drop by drop. The appearance of green or bluish-green indicates the presence of bilirubin in the faeces.

### (5) TEST FOR TRYPSIN

#### *X-ray or Photographic Plate Method (Harrison)*

*Principle.*—Trypsin in alkaline medium will rapidly digest gelatine at body temperature.

*Reagents.*—1. One per cent sodium carbonate.

2. A strip of X-ray or photographic plate.

*Procedure.*—Make a suspension of faeces (1 in 10) in 1 per cent sodium carbonate in a test tube. Place one drop of the suspension on the film side of a strip of X-ray or photographic plate and also a drop of sodium carbonate as a control. Incubate for 30 minutes at 37°C. In order to prevent evaporation of the drops the slip is placed on a piece of moistened filter paper and is covered with the lid of a Petri dish. At the end of this period remove the strip from the incubator and allow to cool to room temperature and then flood the film with cold water. If trypsin be present the gelatine is digested and a clear punched out hole is made in the film. The control area is not dissolved.

*Clinical value of the test.*—The presence of trypsin in the faeces excludes complete obstruction of the pancreatic duct but a negative result is not conclusive evidence that pancreatic secretion is failing to enter the intestine. The faeces of most normal infants contain trypsin and if this enzyme is found in normal amounts in the faeces, fibro-cystic disease of the pancreas can usually be excluded.

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*Lugol's Solution*

4 g. iodine, 6 g. potassium iodide, 100 ml. water.

*Nippé's Reagent*

0.1 g. each of potassium chloride, iodide and bromide in 100 ml. of glacial acetic acid.

*Phenolphthalein*

1 per cent solution in 95 per cent alcohol.

*Phosphate Buffer Solution*

This is made by mixing 15 ml. of Sorensen's solution A with 85 ml. of solution B. To prepare solution A, dissolve 23.88 g. of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  in one litre of boiled distilled water. The solution should be kept in a paraffined bottle. To prepare solution B, dissolve 9.078 g. of  $\text{KH}_2\text{PO}_4$  in a litre of boiled distilled water. This solution should also be kept in a paraffin-coated bottle. The pH of the mixed solutions should be 6.1, which should be confirmed by the hydrogen electrode.

*Potassium Ferricyanide*

Saturated solution made by rubbing crystals in mortar with cold water.

*Silver Nitrate Solution*

29.042 g. silver nitrate in one litre of distilled water. Each millilitre of this solution is equivalent to 0.01 g. of NaCl, or to 0.006 g. of chlorine.

*Salicyl-Sulphonic Acid*

Saturated solution.

*Sodium Hydrosulphite*

10 per cent.

*Sodium Hypobromite*

Dissolve 100 g. of sodium hydroxide in 250 ml. of water. Cool. Now cautiously add 25 ml. of bromine, cooling thoroughly from time to time. For clinical purposes, when the reagent is only occasionally used, it is convenient to purchase the bromine in capsules. 25 ml. of 40 per cent NaOH are measured into a bottle and a bromine capsule containing 2.5 ml. is dropped in and the

bottle immediately stoppered and shaken. The solution must be recently prepared and should be thoroughly cooled before use.

### *Sodium Sulphate (Acid) Solution*

15 per cent solution of sodium sulphate to which acetic acid is added in the proportion of 0.1 ml. to each 100 ml. solution immediately before use.

### *Sodium Thiosulphate Standard N/10 Solution*

Weigh out 25 g. of ordinary sodium thiosulphate. Dissolve in water and dilute to 1 litre. Boiled distilled water must be used. This solution is standardized against an N/10 solution of potassium bichromate.

### *Takayama's Reagent*

This is prepared by mixing sodium hydroxide (10 per cent) 18 ml., saturated glucose solution 18 ml., pyridine 18 ml., and water to 100 ml.

### *Töpfer's Reagent (Dimethylamidoazobenzol)*

0.5 per cent solution in 95 per cent alcohol.

## REAGENTS FOR FOLIN AND WU'S BLOOD ANALYSES

### (A) PREPARATION OF PROTEIN-FREE FILTRATE

1. *10 per cent sodium tungstate.*
2. *2/3N sulphuric acid.*

### (B) ESTIMATION OF CREATININE

1. *Alkaline picrate solution.*—To 25 ml. of saturated solution of picric acid add 5 ml. of 10 per cent NaOH. This must be made freshly on each occasion.

2. *10 per cent NaOH.*

3. *Saturated aqueous solution of pure picric acid.*

4. *Standard creatinine solution.*—Dissolve 1.61 g. of creatinine zinc chloride in sufficient decinormal HCl to make one litre. This contains 1 mg. creatinine per ml. Take 6 ml. of this solution, add 10 ml. decinormal HCl and dilute to one litre.

## REAGENTS FOR ESTIMATION OF BLOOD CHLORIDES (WHITEHORN)

1. *Silver nitrate solution.*—Dissolve 2.905 g. of  $\text{AgNO}_3$  in dis-

tilled water. Transfer to a litre flask and make up to the mark. 1 ml.=1 mg., NaCl.

2. *Potassium thiocyanate solution*.—Dissolve 1.7 g. of potassium thiocyanate in a litre of water and adjust the strength by titration and dilution so that 5 ml. are equivalent to 5 ml. of the  $\text{AgNO}_3$  solution.

3. *Powdered ferric ammonium sulphate (alum)*.

4. *Nitric acid (pure)*.

#### REAGENTS FOR ESTIMATION OF PHOSPHATE CONTENT OF PLASMA (Method of Fiske and Subbarow)

1. *Ammonium molybdate No. 1*.—Dissolve 25 g. of the salt in 200 ml. of water. Rinse into a litre flask containing 500 ml. of 10N sulphuric acid, dilute to the mark with water and mix.

2. *Ammonium molybdate No. 2*.—Dissolve 25 g. of the salt in 200 ml. of water. Rinse into a litre flask containing 300 ml. of 10N sulphuric acid, dilute to the mark with water and mix.

3. *Aminonaphtholsulphonic acid*.—Dissolve 0.5 g. of the dry powder in 195 ml. of 15 per cent sodium bisulphite. Now add 5 ml. of 20 per cent sodium sulphite, stopper and shake thoroughly until dissolved.

4. *Standard phosphate solution*.—Dissolve 0.3509 g. of pure monopotassium phosphate in water. Transfer to a litre volumetric flask and add 10 ml. of 10N sulphuric acid. Dilute to the mark and mix. For use dilute 1 in 10 (5 ml.=0.04 mg. P).

#### REAGENTS FOR BENEDICT'S METHOD OF URIC ACID ESTIMATION

1. *Sodium cyanide*.—A 5 per cent solution containing 2 ml. of concentrated ammonia per litre. Prepared fresh every two months.

2. *Uric acid reagent*.—100 g. of pure sodium tungstate are placed in a litre flask and dissolved in about 600 ml. of water. 50 g. of pure arsenic pentoxide are now added, followed by 25 ml. of 85 per cent phosphoric acid, and 20 ml. of concentrated hydrochloric acid. The mixture is boiled for 20 minutes, cooled, and diluted to one litre. The reagent keeps indefinitely.

3. *Uric acid standard solutions*.—The stock solution is made as follows: Dissolve 18 g. of pure crystallized disodium hydrogen

phosphate ( $12\text{H}_2\text{O}$ ) and 1 g. crystallized sodium dihydrogen phosphate in 200 to 300 ml. of hot water. Filter, if not perfectly clear. Make up to about 500 ml. with hot water and pour this hot, clear solution upon exactly 200 mg. pure uric acid suspended in a few ml. of water in a litre volumetric flask. Agitate a few minutes until the uric acid completely dissolves. Cool. Add *exactly* 1.4 ml. of glacial acid, dilute to mark, and mix. Add about 5 ml. of chloroform to prevent the growth of bacteria or moulds in the solution. Five ml. of this solution contains exactly 1 mg. of uric acid. This solution should keep about two months, if in a cool room.

The dilute standard containing 0.02 mg. of uric acid in 5 ml. is prepared as follows: 10 ml. of the stock uric acid phosphate solution are measured into a 500 ml. volumetric flask and the flask is about half-filled with distilled water. 25 ml. of dilute HCl (one volume of concentrated acid diluted to 10 volumes with water) are added, and the solution is diluted to 500 ml. This solution should be prepared fresh every two weeks, as it is unstable.

## REAGENTS FOR ESTIMATION OF "ALKALINE" PHOSPHATASE OF BLOOD SERUM

(Method of King and Armstrong)

1. *Buffer*.—M/10 sodium carbonate—bicarbonate 6.36 g. anhydrous sodium carbonate and 3.36 g. sodium bicarbonate are dissolved in distilled water and made to one litre.

2. *Substrate*.—M/100 disodium phenyl phosphate 2.18 g. dissolved in one litre of water. The solution should be brought quickly to the boil to destroy any organisms, cooled immediately and preserved with a little chloroform.

3. *Buffer substrate*.—Mix equal quantities of buffer and substrate to give a final concentration of M/200 disodium phenyl phosphate and M/20 sodium carbonate—bicarbonate buffer.

4. *Phenol reagent*.—Dissolve 100 g. sodium tungstate and 25 g. sodium molybdate in 700 ml. water in a 1,500 ml. flask. To this add 50 ml. of syrupy 85 per cent phosphoric acid and 100 ml. concentrated hydrochloric acid. The flask is now connected with a reflux condenser, using a rubber stopper wrapped in tinfoil.

The solution is then boiled for 10 hours. 150 g. lithium sulphate, 50 ml. of water and a few drops of bromine are now added and the mixture boiled without a condenser for about a quarter of an hour. It is then cooled and diluted to 1 litre. For use this reagent is diluted 1 in 3.

5. *20 per cent solution of sodium carbonate* ( $\text{Na}_2\text{CO}_3$ ).

6. *Stock standard phenol*.—Dissolve 1 g. crystalline phenol in N/10 HCl and make up to 1 litre with N/10 HCl. Transfer 25 ml. of this solution to a 250 ml. flask, add 50 ml. N/10 sodium hydroxide and heat to 65°C. 25 ml. of N/10 iodine solution is added to the hot solution, the flask is stoppered and allowed to stand at room temperature for half to three-quarters of an hour. 5 ml. of concentrated hydrochloric acid is now added and the excess of iodine titrated with N/10 thiosulphate solution. Each ml. of N/10 iodine corresponds to 1.567 mg. of phenol.

7. *Diluted stock standard phenol*.—Exactly 10 mg. phenol per 100 ml. solution. Made by a suitable dilution of 6. This keeps at least three months in the refrigerator.

8. *Standard phenol solution and reagent*.—1 mg. phenol per 100 ml.:

5 ml. diluted stock standard (10 mg. per 100 ml.).

15 ml. diluted phenol reagent.

Water to 50 ml.

(Make up daily).

#### REAGENTS FOR ESTIMATION OF "ACID" PHOSPHATASE OF BLOOD SERUM

(Method of Gutman and Gutman)

1. *Buffer-substrate* (0.005M monophenyl phosphate, 0.1M citrate at pH 4.9).

*Solution A*. Dissolve 1.09 g. of disodium phenyl phosphate in 500 ml. of water.

*Solution B*. Dissolve 42.0 g. of crystalline citric acid in water, add 376 ml. of 1N NaOH, make up to 1 litre.

Mix equal parts of solution A and B as needed. Adjust the pH to 4.9 with NaOH or HCl (when colorimetric methods are used, nitrazine (Squibb) is a convenient indicator). Preserve in well-stoppered bottles in a refrigerator.

2. *Phenol reagent of Folin and Ciocalteu*.—The stock reagent is prepared as described by Folin and Ciocalteu, kept in a well-stoppered amber bottle and for use diluted 1:3.

3. *Sodium Carbonate* (20 per cent solution).

4. *Standard phenol*.—For the stock solution, which keeps indefinitely, dissolve 1 g. of crystalline phenol in 0.1N HCl and make up to one litre with 0.1N HCl. Standardize by the method of Koppeschaar. From this stock phenol solution a diluted phenol solution containing *exactly* 10 mg. of phenol per 100 ml. is made up; it remains stable for months in a refrigerator.

5. *Standard phenol solution and reagent*.—To 1 ml. of diluted phenol solution in a test tube, add 6 ml. of distilled water and 3 ml. of diluted phenol reagent. Prepare shortly before use.

#### REAGENTS FOR HANGER FLOCCULATION TEST

*Cephalin*.—A satisfactory sample can be prepared by the method given by Hanger (*J. Clin. Invest.*, 18, 261, 1939).

*Cephalin-cholesterol stock solution*.—100 mg. cephalin and 300 ml. of cholesterol are dissolved in 8 ml. of peroxide-free ether. This solution will keep for a considerable period if stored in a refrigerator.

*0.85 per cent saline* is prepared, using freshly distilled water and reagent quality sodium chloride.

*Cephalin-cholesterol emulsion*.—35 ml. of freshly distilled water are heated to 65-70°C. One ml. of stock solution is added slowly drop by drop with constant stirring. This addition completed the mixture is heated slowly to boiling and is then simmered gently until the volume is reduced to 30 ml. Cool before use.

#### REAGENTS FOR ESTIMATION OF POTASSIUM IN SERUM

(Method of Kramer and Tisdall)

1. *Sodium cobaltinitrite reagent*.

(A) 25 g. of cobalt nitrate crystals are dissolved in 50 ml. of water; 12.5 ml. glacial acetic acid is then added.

(B) 120 g. of sodium nitrite (K free) are dissolved in 180 ml. water.

To all of solution A are added 210 ml. of solution B. Air is

drawn through the solution until all the gas has passed off (it will still smell of nitrous oxide and be a red yellow). Store in refrigerator and filter before use. It will keep for three months.

2. *N/100 potassium permanganate*. — See description under standard solution.

3. *5N sulphuric acid*.

4. *N/100 sodium oxalate*.—5 ml. N/10 sodium oxalate—1 ml. N/10  $\text{H}_2\text{SO}_4$  and water to 50 ml. Titrate against N/100  $\text{KMnO}_4$ .

5. *Standard potassium solution*.—8.914 g.  $\text{K}_2\text{SO}_4$  per litre containing 4 mg. K per ml. Dilute 10 ml. to 100 ml.

N.B.—Use serum obtained less than 1 hour after removal from patient. A dry syringe must be used.

#### REAGENTS FOR ESTIMATION OF SODIUM IN BLOOD SERUM (Method of Weinbach)

1. *Uranyl zinc acetate reagent*.

*Solution A*.—77 g. of uranyl acetate,  $\text{UO}_2(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ , and 14 ml. of glacial acetic acid are dissolved by gently heating and stirring in about 400 ml. of water and diluted to 500 ml. in a volumetric flask.

*Solution B*.—231 g. of zinc acetate  $\text{Na}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$ , and 7 ml. of glacial acetic acid are dissolved by gently heating and stirring in about 400 ml. of water and diluted to 200 ml. in a volumetric flask.

The two solutions A and B are mixed, while hot, allowed to stand 24 hours or longer and filtered.

2. *Acetone wash reagent*.

A small amount of triple salt, uranyl zinc sodium acetate, is prepared by adding 15 ml. of the uranyl zinc acetate reagent to 1 ml. of an approximately 5 per cent solution of  $\text{NaCl}$  with subsequent addition of about 5 ml. of 95 per cent alcohol in small portions. Filter with suction and wash the pipette with 4 or 5 portions of 95 per cent alcohol and then 4 or 5 portions of ether, sucking dry after each addition of alcohol or ether. Add this amount of triple salt to 1 litre of acetone, shake, let stand over night, filter.

### 3. *Standard sodium solution.*

Exactly 1 g. of NaCl is dissolved in water and made up to a litre in a volumetric flask. Each ml. of this solution contains 0.393 mg. of Na and 0.5 ml. is equivalent to exactly 3.42 ml. of 0.02N NaOH.

## REAGENTS FOR ESTIMATION OF BLOOD SERUM PROTEINS

### (Method of Weichselbaum)

#### *Biuret reagent (Weichselbaum)*

1.5 g. Copper sulphate.

6.0 g. Sodium potassium tartrate.

1.0 g. Potassium iodide.

750 ml. 1.0N sodium hydroxide (final concentration of sodium hydroxide 3 per cent).

*Procedure.*—Dissolve copper sulphate in approximately 50 ml. distilled water. Dissolve sodium potassium tartrate and potassium iodide in 50 ml. distilled water and add to copper sulphate solution. Add sodium hydroxide solution slowly to ensure that copper sulphate is completely dissolved. Make up to 1 litre with distilled water.

#### *Standardization of the Biuret reagent (Weichselbaum)*

*Kjeldahl estimations.*—Take 5 ml. of serum, dilute to 100 ml. using a standard flask. Mix thoroughly. Use the following aliquots:

25 ml. of the diluted serum—1.25 ml. of undiluted serum.

20 ml. " " " " —1.0 ml. " " "

15 ml. " " " " —0.75 ml. " " "

10 ml. " " " " —0.50 ml. " " "

5 ml. " " " " —0.25 ml. " " "

Do Kjeldahl estimation in the usual way.

#### *Colorimetric standardization*

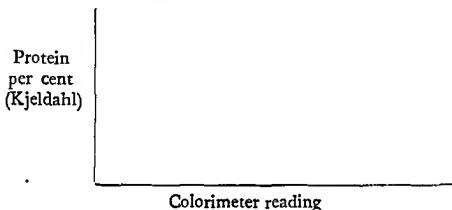
Since in the Biuret method for A/G ratio a volume equivalent to 0.1 ml. of serum is taken for each test, similar dilutions are used for colorimetric determination of the total protein.

Take 5 ml. of the same serum as was used for the Kjeldahl and dilute to 50 ml. Mix thoroughly.

Take aliquots:

Serum (1/10)	Saline
(5) 0.25 ml. diluted with 1.75 ml.	
(4) 0.50 ml.     "     "	1.50 ml.
(3) 0.75 ml.     "     "	1.25 ml.
(2) 1.00 ml.     "     "	1.00 ml.
(1) 1.25 ml.     "     "	0.75 ml.

The colorimeter readings are then plotted on graph paper versus the values obtained by the Kjeldahl estimations.



A report on one complete total protein and albumin-globulin ratio can be obtained in 1 hour. Half of this time is occupied by incubation and can be utilized for other work. Several tests could run in one and a half hours.

#### REAGENTS FOR ESTIMATION OF TOTAL SERUM PROTEIN

(Method of Phillips, *et al.*)

##### *Preparation of stock copper sulphate*

Solution of specific gravity 1.1000.

Dissolve 159.0 g. of copper sulphate AR in water. Make up to one litre in volumetric flask. The temperature of the copper sulphate solution must be 25°C when it is made up to the litre mark on the flask. This solution should be tested with a hydrometer to determine that the specific gravity is 1.1000.

##### *Preparation of sets of standard solutions*

A series of stoppered bottles of 150-200 ml. capacity is obtained; preferably wide mouth bottles with ground glass stoppers which are lightly brushed with vaseline so as to ensure

that the stoppers are airtight. Stock copper sulphate solution of specific gravity 1.1000 is placed in a burette and run into a volumetric flask, the flask is then filled to the mark with water at 25°C, shaken well and the contents placed in one of the wide mouth bottles with the appropriate label. These solutions if kept stoppered except for the few minutes of use will keep for some 100-200 estimations. If the copper sulphate has any contamination with iron, a yellow deposit will form during the next few weeks but this is usually not sufficient to affect the solution. Table XXXVII shows the amount of copper sulphate solution required for 100 ml. of the standard solutions of known specific gravity. A litre of each dilution may be made up and stored in stock bottles.

TABLE XXXVII

Cc. of Stock Copper Sulphate Solution of Gravity 1.1000 to be diluted to 100 ml. to Prepare Standard Solutions of Gravity, G, to within  $\pm 0.0001$

G	100	G	100	G	100
1.008	7.33	1.031	30.0	1.054	53.0
09	8.32	32	31.0	55	54.0
10	9.31	33	32.0	56	55.0
11	10.30	34	33.0	57	56.0
12	11.29	35	34.0	58	57.0
13	12.28	36	35.0	59	58.0
14	13.27	37	36.0	60	59.0
15	14.26	38	37.0	61	60.0
16	15.25	39	38.0	62	61.0
17	16.24	40	39.0	63	62.0
18	17.23	41	40.0	64	63.0
19	18.22	42	41.0	65	64.0
20	19.21	43	42.0	66	65.0
21	20.20	44	43.0	67	66.0
22	21.19	45	44.0	68	67.0
23	22.17	46	45.0	69	68.1
24	23.15	47	46.0	70	69.1
25	24.14	48	47.0	71	70.2
26	25.12	49	48.0	72	71.2
27	26.10	50	49.0	73	72.2
28	27.08	51	50.0	74	73.3
29	28.06	52	51.0	75	74.3
30	29.04	53	52.0		

G = specific gravity of standard solution.

N.B.—If for example the solution of specific gravity of 1.008 is desired one measures 7.33 ml. of stock solution and dilutes it to 100 ml

## APPENDIX B

### SUMMARY OF METHOD OF QUALITATIVE EXAMINATION OF PATHOLOGICAL URINE

1. *Note the colour, odour, clarity, or turbidity, specific gravity and reaction.*

2. *Examine urinary sediment.*

The urine should be centrifuged or allowed to sediment in a tall glass cylinder—toluol being used as a preservative. Abnormal constituents may include various types of crystals, casts, epithelial cells, leucocytes, erythrocytes, animal parasites, micro-organisms, and foreign debris.

3. *Test for albumin.*

(a) Heat and acetic acid test.

(b) Three tubes test for albumin or mucin

(c) Salicyl-sulphonic acid test.

4. *Test for sugar.*

(a) Benedict's test.

(b) Phenylhydrazine test.

(c) Fermentation test.

If albumin be present, it should first be removed by acidifying, boiling the urine, filtering and neutralizing the filtrate.

5. *Test for aceto-acetic acid.*

(a) Ferric chloride test.

(b) Rothera's test (also a test for acetone).

6. *Test for acetone.*

Rothera's test.

7. *Test for blood and blood pigments.*

(a) Chemical (benzidine) test.

(b) Spectroscopic examination.

(c) Microscopic examination.

8. *Test for bile pigments.*

(a) Fouchet's test.

(b) Cole's test.

9. *Test for urobilin and urobilinogen*

- (a) Bogomolov's test.
- (b) Schlesinger's test.
- (c) Ehrlich's aldehyde test.

10. *Test for bile salts.*

Hay's sulphur test.

## SUMMARY OF METHOD OF EXAMINATION OF GASTRIC CONTENTS

## (1) QUALITATIVE

1. *Test for presence of free HCl.*

Günzberg's test.

2. *Test for pepsin.*

Fibrin test.

3. *Test for lactic acid.* (If free HCl be absent.)

(a) Ferric chloride test.

(b) Uffelmann's test.

4. *Test for blood.*

Benzidine test.

5. *Test for starch.*

Iodine test.

6. *Test for bile pigment.*

(a) Fouchet's test.

(b) van den Bergh's test.

7. *Examine gastric sediment*—particularly that of the resting gastric contents. Erythrocytes, pus cells, food residues from previous meals, shreds of tissue and micro-organisms may be noted.

## (2) QUANTITATIVE

1. Estimate free hydrochloric acid.

2. Estimate total acidity.

## SOME USEFUL WEIGHTS AND MEASURES

1 gram	=15.432 grains.
1 microgram	=0.001 mg.
1 kilogram	=2.2046 lbs.
1 ml.	=16.9 minims.
1 litre	=35.2 fluid ozs.=1.76 pints.
1 pint	=20 fluid ozs.=567.9 ml.
1 gallon	=8 pints=4.542 litres.

Osmotic pressure of molar solution (undissociated)  
=22.4 atmospheres.

Normal solution=one gram-equivalent of reagent per litre.

## APPENDIX C

### QUALITATIVE VAN DEN BERGH REACTION

#### (1) DIRECT REACTION

*Principle.*—Ehrlich's diazo reagent used in the van den Bergh test gives a colour reaction with bilirubin.

*Reagents.*—1. Ehrlich's diazo reagent (p. 357).

*Procedure.* — Take three tubes and add equal volumes (0.25 ml.) of serum to each. To (a) add 0.2 ml. of water, to (b) add 0.2 ml. fresh diazo reagent and let stand 10 minutes or until the maximum colour is developed, and to (c) at the end of 10 or 15 minutes add 0.2 ml. diazo reagent. Compare this tube (c) with (a) serum control and (b) showing the maximum colour and decide whether the reaction in (c) is immediate, biphasic or delayed.

The test should be carried out no longer than two hours after collecting the blood. Great care should be exercised in having the glassware clean, as acid or alkali markedly affect the colour of the azobilirubin compound. Oxalated plasma is used, since haemolysis in a serum interferes with the reaction.

To obtain the plasma, 0.2 ml. of a 10 per cent solution of potassium oxalate is placed in a small loosely-corked bottle and evaporated to dryness during sterilization of the bottle. This oxalate prevents coagulation when 10 ml. of blood obtained from a vein is introduced. The plasma is obtained after centrifuging the blood.

*Types of reaction.*—One of the following reactions may occur :

1. *Immediate direct reaction.*—This begins instantly, and is maximal in 10 to 30 seconds. The colour obtained is a bluish violet of intensity depending upon the amount of bilirubin present.

2. *Delayed direct reaction.*—This begins in 1 to 15 minutes, or even longer, and consists in the development of a reddish colour which gradually deepens and becomes more violet.

3. *Biphasic direct reaction.*—In this reaction a slight reddish colour appears immediately (10 to 30 seconds), which, after a

The logarithmic scale is obtained in the following way. A reading of 100 on the linear scale, i.e. complete transmission of incident light, corresponds to 0 on the logarithmic scale while 10 on the linear is 1 ( $\log \frac{100}{10} = 1$ ) on the logarithmic. Similarly if a coloured solution transmits 50 per cent of the incident light then  $D = \log \frac{100}{50} = 0.301$ . The values of optical density are usually multiplied by 100 so that the values on the scale are expressed as whole numbers.

### *Operation of the instrument*

The instrument is switched on and the shutter is adjusted until the needle is on 0 on the logarithmic scale with either a reagent blank or distilled water in the tube. The former should be used unless the reagents themselves do not absorb light. The blank should be prepared in exactly the same way as the test but distilled water should be used instead of the test solution. The blank is then replaced in turn by the standard and the test and the optical densities of these measured. It is advisable to check frequently the zero setting with the blank.

The concentration of the standard should be fairly close to that of the unknown and the calculation is:

Concentration of unknown =

$$\frac{\text{reading of unknown}}{\text{reading of standard}} \times \text{concentration of standard.}$$

*Note.* Ratio of readings is reversed from that used with visual colorimeters since the reading is directly proportional to concentration here but is inversely proportional in the other case.

Alternatively a standard curve may be constructed in which the optical density of a number of standards is plotted against their concentrations. It is advisable however to run one standard through with the unknown to check variations in the reagents or in the colorimeter.

In most determinations a filter is used and two factors should be considered in choosing an appropriate filter: (1) there should be a linear relationship between optical density and concentration at the wavelength chosen and (2) the gradient of the straight

line so obtained should be great enough to give the required sensitivity. Usually the filter selected is that which allows maximum absorption for the particular colour produced.

The tubes which hold the solutions while reading should be matched, i.e. with the same coloured solution in all tubes the optical density should be the same for each tube.



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